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SPECIES RANGES, HOST SELECTION, AND HYBRIDIZATION: HOW INCREASED HYBRIDIZATION IS LEADING TO HOST USE DIVERGENCE IN A POLYPHAGOUS SIBLING SPECIES PAIR

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SPECIES RANGES, HOST SELECTION, AND HYBRIDIZATION: HOW INCREASED HYBRIDIZATION IS LEADING TO HOST USE DIVERGENCE IN A POLYPHAGOUS SIBLING SPECIES PAIR.

By

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ABSTRACT

SPECIES RANGES, HOST SELECTION, AND HYBRIDIZATION: HOW INCREASED HYBRIDIZATION IS LEADING TO HOST USE DIVERGENCE IN A POLYPHAGOUS SIBLING SPECIES PAIR.

By

Rodrigo J. Mercader

In this dissertation I use the *P. glaucus* and *P. canadensis* system to understand how climate change will affect interactions between organisms. In particular, how increased genetic introgression caused by climate change may alter plant-insect associations.The sibling species *Papilio glaucus* and *Papilio canadensis* meet in a narrow hybrid zone believed to be maintained by temperature thresholds acting independently on both species. However, due to recent climate change an increased movement of *Papilio glaucus* into historically *P. canadensis* territory has been observed, which has lead to greater genetic introgression (Scriber 2002), including the formation of a delayed emerging phenotype or "late flight" first observed in 1999 (Scriber and Ording 2005; Scriber et al. 2008). To understand how climate change will affect future interactions between these butterflies I first tested the assumption that the *P. canadensis* range is limited by rare high temperatures in late summer and/or autumn on pupal survival. Short periods of high temperatures did not induce the high mortality rates required to be the key factor limiting the range of *P. canadensis*. However, *P. canadensis* did exhibit a considerably lower tolerance to high temperature extremes and conditions simulating shorter/warmer winters than *P. glaucus*. Subsequently, I tested how increased genetic introgression will likely affect a central interaction in plant feeding insects, host use. To

accomplish this I first investigated what the difference in host use between *P. glaucus* and *P. canadensis* is. The results from this investigation indicated that the primary difference between *P. glaucus* and *P. canadensis* is limited to a Z-linked shift in host rank hierarchy due to an acceptance of *Populus tremuloides* Michx. (Salicaceae) and reduced specificity for *Liriodendron tulipifera* L. (Magnoliaceae) in *P. canadensis.* Finally, I assessed how the recombination of the parental genomes leading to the formation of the "late flight" phenotype may have facilitated another major ecological shift, host use divergence. The results of this investigation indicate that the ovipositional preference of this hybrid swarm is identical to that of the introgressing parental species, *P. glaucus*. Due to the absence of the preferred hosts of *P. glaucus* where the "late flight" occurs, this ovipositional pattern implies an apparent functional shift onto a secondary host of both parental species, *F. americana*. In contrast, the larval host use abilities represent a mixture of *P. glaucus* and *P. canadensis*, indicating divergence in larval host use abilities has not taken place. However, high genetic variability (CV_G) is present for growth on *F. americana* and tradeoffs for larval performance on the preferred hosts of the two parental species are evident; indicating a strong potential for specialization in larval host use abilities to occur. This current scenario represents an instance where a shift in a major ecological trait, host use, is likely occurring as a byproduct of a shift in an unrelated trait (delayed emergence) leading to partial reproductive isolation.

To Becky Mercader

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION

The recent environmental changes taking place have highlighted our need to better understand how interactions between organisms evolve. Rapid environmental changes have not only lead to population extinctions, but the novel selection pressures placed upon organisms have lead to several examples of rapid evolution (e.g. examples reviewed in Thompson 1998; Palumbi 2001). These instances of rapid evolution have included well documented shifts in the interactions between plant feeding insects and their hosts due to plant invasions (Strauss et al 2006; Carroll 2007; Singer *et al.*2008) and land use change (Singer *et al.*2008). However, little is known about how climate change may affect plant-insect associations, other than through local extinctions. In particular, how increased gene flow may affect host use traits and alter the associations between plant and insects. This issue is of particular importance in the Lepidoptera as hybridization is very common in this group, occurring in > 15% of all species (Sperling 1990; Presgraves 2002). Global climate change has already affected the geographic ranges of numerous species, with warming most often resulting in shifts in species distributions of insects toward higher latitudes or altitudes (Thomas *et al.*2001; Hill *et al.*2002; Parmesan and Yohe 2003; Root *et al.*2003; Walther 2005; Parmesan 2006), thus greatly increasing the likelihood and/or rate of hybridization.

Due to their high diversity, plant feeding insects have been the focus of multiple studies on speciation and adaptive radiation. This high diversity of plant feeding insects has often been attributed to their diversification on the wide chemical diversity of plants

(e.g. Ehrlich & Raven, 1964; Mitter *et al.,* 1988; Futuyma, 1989; Weingartner *et al.,* 2006). Although divergence through specialization has often been considered a primary mechanism for diversification in plant feeding insects, recent reviews have indicated a high level of transition from specialist to generalist phases in insect lineages (Janz *et al.*2001; Nosil 2002). In addition, as described by Janz *et al.* (2006) diversification through specialization alone would quickly reach a dead end as lineages become increasingly specialized. Instead, Janz *et al.*(2006) propose a hypothesis whereby insect lineages oscillate between specialized and generalized forms, in which initial divergence occurs in the generalized phase and subsequent specialization leads to greater diversification.

Relatively little is known about how host shifts occur during polyphagous stages of insect lineages, as the vast majority of this work has been conducted on monophagous or oligophagous species (e.g., Carroll & Boyd, 1992; Radtkey & Singer, 1995; Menken, 1996; Feder *et al.*, 1998; Abrahamson *et al.*, 2001; Hora *et al.*, 2005; Ohshima & Yoshizawa, 2006; Stastny *et al.*, 2006; but see Thompson, 1998; Janz *et al.*, 2001 for shifts between specialized and generalized forms). This bias is likely a reflection of the greater number of specialist insects and the relatively easier task of detecting host shifts in specialized insects.

The polyphagous and widely distributed species of the *Papilio glaucus* group are probably the most widely studied non-pest generalist insect group, particularly the sibling species *Papilio glaucus* and *Papilio canadensis.* The large ecological knowledge base we have for these insects makes them an ideal model system to study how climate warming will affect the interactions between plant feeding insects and their host plants. These two

sister species share many ecological similarities, can readily produce fertile hybrid offspring (e.g. Scriber 1998), and until recently were considered the same species (Hagen *et al.*1991). However, despite their similarities they exhibit significant differences in host plant use. In particular, tulip tree, *Liriodendron tulipifera* (Magnoliaceae), the preferred host of *P. glaucus,* is toxic to *P. canadensis* larvae, while quaking aspen, *Populus tremuloides* (Salicaceae), the preferred host of *P. canadensis*, is toxic to *P. glaucus.* Historically these two species have had a narrow, but extensive, hybrid zone occurring at the ecotone between temperate deciduous and boreal forests. Recent climate change has lead to an increased emigration of *P. glaucus* into historically *P. canadensis* territory, which in turn has lead to increased hybridization between the two species. This has created an allochronically separated hybrid swarm population (Scriber and Ording 2005, Scriber *et al.*2008).

In this dissertation I use the *P. glaucus* and *P. canadensis* system as a model system to understand how climate change is likely to affect the interaction between organisms. In particular, I explore how increased genetic introgression caused by climate change may alter plant-insect associations. I do this by first testing the factors limiting the distribution of these insects (Chapter 2), then identifying the factors underlying the differences in host selection between these two species (Chapter 3), and finally by assessing the host preference and performance of the hybrid swarm population produced by the genetic introgression of *P. glaucus* into *P. canadensis* (Chapter 4).

INTRODUCTION TO THE SYSTEM

The Eastern Tiger Swallowtail, *P. glaucus* (L.) and the Canadian Tiger Swallowtail, *P. canadensis* are generalists in both the number of tree species they consume (30-40 species in 14 plant families; Scriber 1988) and in the temperature regimes they experience (combined ranges extend from Southern Florida U.S.A. to Central Alaska U.S.A.). The Northern range limit of *P. glaucus* coincides with the Southern range limit of *P. canadensis* in a narrow but extensive hybrid zone at the ecotone between boreal and temperate deciduous forests (Scriber *et al.*2003). This zone coincides with the thermal limit for the completion of two generations (1556-1611 C Degree days base 10 °C), and not surprisingly marks the range of the facultative diapauser *P. glaucus* with a minimum of two generations per year (Scriber 1996). This hybrid zone also delimits the Southern range of the obligate diapauser *P. canadensis,* which is limited in part by lower pupal survival in warmer conditions (Scriber *et al.*2002; Mercader and Scriber 2008a) as well as likely interactions with *P. glaucus* and shifts in host plant availability.

Despite being highly polyphagous, *P. glaucus* and *P. canadensis* have strong ovipositional preferences for particular hosts, including local adaptation in ovipositional specificity in both species (Bossart and Scriber 1995; Scriber 1996b, 2002b), and significant differences between the two species (Scriber *et al.*1991, 1995, 2003; Mercader and Scriber 2007). In addition, they exhibit significant differences in their growth rates on different hosts, including significant differences between each other (Reviewed in Scriber *et al.*1995; Scriber 1996; Scriber *et al.*2003). In particular, the preferred host of *P.*

glaucus, tulip tree, *Liriodendron tulipifera* L. (Magnoliaceae), is toxic to the larvae of *P. canadensis*, whereas one of the most common hosts of *P. canadensis,* quaking aspen, *Populus tremuloides* Michx. (Salicaceae), is toxic to the larvae of *P. glaucus*. Additionally, larval host use abilities differ significantly for some secondary hosts such as spicebush, *Lindera benzoin* (L.) Blume (Lauraceae), which is a marginal host for *P. glaucus* and toxic for *P. canadensis* and paper birch, *Betula papyrifera* Marshaff (Betulaceae), which is an excellent host for *P. canadensis,* but only marginal for *P. glaucus.* Despite these differences, these two species do have multiple secondary hosts in common. Among these are white ash, *Fraxinus americana* L. (Oleaceae), black cherry, *P. serotina*, and Hop tree, *Ptelea trifoliata* L. (Rutaceae) [note: *P. canadensis* will rarely encounter *P. trifoliata*], which are all excellent hosts for the larvae of *P. canadensis* and *P. glaucus.* In addition, adult female ovipositional preferences for these secondary hosts are essentially identical (Mercader and Scriber 2007).

A recent increase in immigration of *P. glaucus* into historically *P. canadensis* territory due to climate warming has lead to increased hybridization between these two species. This has resulted in the formation of a univoltine allochronically separated hybrid swarm population (Scriber and Ording 2005, Scriber *et al.*2007). This hybrid swarm has been noted, at the population level, to have larvae survive on both *L. tulipifera* and *P. tremuloides* (Scriber *et al.*2007), and three choice oviposition bioassays indicate its ovipositional behavior may follow that of *P. glaucus* (Mercader and Scriber 2007).

CHAPTER 2

Mercader RJ, Scriber JM (2008) Asymmetrical thermal constraints on the parapatric species boundaries of two widespread generalist butterflies. Ecological Entomology (In Press).

ASYMETRICAL THERMAL CONSTRAINTS ON THE PARAPATRIC SPECIES BOUNDARIES OF TWO WIDESPREAD GENERALIST BUTTERFLIES.

Abstract

The sibling species *Papilio glaucus* and *Papilio canadensis* meet in a narrow hybrid zone believed to be maintained by temperature thresholds acting independently on both species. Here we test if this assertion is true for the cold adapted species, *P. canadensis,* which is presumed to be limited by rare high temperatures in late summer and/or autumn on pupal survival. Three experiments were conducted examining the effects of 1) short periods of high temperature stress in autumn, 2) prolonged warm temperatures in autumn, and 3) temperatures simulating warmer winters/longer springs upon the survival of *P. canadensis* and *P. glaucus.* Results indicated that short periods of high temperatures did not induce the high mortality rates required to be the key factor limiting the range of *P. canadensis*. However, *P. canadensis* did exhibit a considerably lower tolerance to high temperature extremes and conditions simulating shorter/warmer winters than *P. glaucus*. In combination, differences in temperature tolerance throughout the pupal stage are likely to be a significant factor in maintaining the southern range limit of *P. canadensis*. Further warming as may occur during climate change, particularly in winter and spring, will likely affect the dynamics of southerly populations of *P. canadensis.*

Introduction

The factors determining species ranges have been a central focus of ecological research, as they represent the thresholds of tolerance for organisms to biotic and abiotic factors. Current changes in global climate patterns present a new challenge to organisms as increases in global temperature are likely to increase the likelihood of both population extinctions (e.g. McLaughlin *et al.*, 2002; Hoyle & James, 2005; Shoo *et al.*, 2005) and species range expansions (e.g. Parmesan *et al.*, 1999; Thomas *et al.*, 2001; Walther *et al.*, 2002; Crozier, 2004; Battisti *et al.*, 2005). These shifts are likely to drastically alter the species composition of regions, particularly those at ecotones where many range limits overlap. Therefore, as the abiotic and biotic environment change, our ability to predict shifts in insect community composition will depend on our understanding of the factors that limit their ranges.

The environmental factors that define range limits in organisms, and the mechanisms through which they act are necessary in order to understand how species distributions may change. For temperate insects two major environmental constraints are the length of the growing season and large fluctuations in temperatures throughout the year. Constraints in the length of the growing season can be seen in the number of organisms exhibiting geographical variation in the number of generations per year (Tauber & Tauber, 1981; Ayres & Scriber, 1994). Constraints imposed by fluctuating temperatures can be seen in the prolonged periods of inactivity most insects undergo in stages that can tolerate environmental extremes (e.g. diapausing pupae). These adaptations allow many species to maintain extensive geographic ranges, often with locally adapted populations differing in their thresholds for diapause induction, duration

of diapause, and rate of diapause and non-diapause development (Tauber &Tauber, 1981). The factors that define the ranges of these widespread species are likely to signify important physiological and/or ecological tradeoffs.

The Eastern tiger swallowtail, *Papilio glaucus* (L.) and the Canadian tiger Swallowtail, *Papilio canadensis* (R & J) (Lepidoptera: Papilionidae, these *Papilio=Pterourus*) are extreme generalists in both the number of tree species they consume (30-40 species in 14 plant families; Scriber, 1988) and in the temperature regimes they experience. Their extensive ranges, which combined extend from Southern Florida to Central Alaska U.S.A., cover a vast range of biotic and abiotic conditions. The Northern range limit of *P. glaucus* coincides with the Southern range limit of *P. canadensis* in a narrow but extensive hybrid zone at the ecotone between boreal and temperate deciduous forests (Scriber, 1994). While the northern range limit of *P. glaucus* is defined by the thermal environment that allows the completion of two generations (Scriber, 1994), the factors determining the southern range limits of the univoltine *P. canadensis* are not clearly understood.

The first flight of *P. glaucus* and the only flight *P. canadensis* occur as spring begins, and by late May the flights of both species co-occur near the hybrid zone (and potentially from the central United States to Alaska; Scriber 1996). Although the flights of both species co-occur and both overwinter as pupae, *P. glaucus* has two or more generations per summer and is therefore less likely to encounter high temperatures in an overwintering pupal stage than the univoltine *P. canadensis.* Scriber *et al.*(2002) found extremely high mortality rates (near 100%) of *P. canadensis* and *P. glaucus* pupae exposed to high temperatures during emergence in spring (30, 33, & 36 ° C). The low

tolerance observed for high temperature in *P. canadensis* pupae would pose a strong limiting factor on the southern range of *P. canadensis* even if high temperature extremes were relatively rare. This lead Scriber *et al.*(2002) to conclude that rare periods of extreme high temperatures during late summer or early Autumn were the key factor determining the southern range limit of *P. canadensis* (Scriber *et al.*, 2002). Extreme temperature events have been increasing and are expected to continue increasing (e.g. Easterling *et al.*, 2000; Clark *et al.*, 2006) and *P. canadensis* populations above the hybrid zone may begin to encounter such conditions with greater frequency. If rare periods of extreme high temperatures are the key factor limiting the range of *P. canadensis,* southern populations of *P. canadensis* could be at risk of local extinction.

However, while summer and autumn temperatures may occasionally be at or near 30° C, spring emergence temperatures at the *P. canadensis* southern range limit are unlikely to reach daily temperature averages above 30° C. For example, in Kalamazoo Michigan (located South of the hybrid zone) between 1948 and 1998 there were no days where the maximum and minimum temperature averaged 28° C or more between early April and early June (KBS, LTER site weather station). In contrast, during the same time period there were 10 separate years in which daily temperatures averaged 28° C or more for at least three consecutive days between late July and the end of September (KBS, LTER site weather station).

The tolerance for extreme temperatures is likely to be significantly higher prior to the onset of winter when diapausing pupae are in a quiescent phase than in the spring just prior to emergence (when the imago is forming inside the pupa). Therefore, in this study we re-evaluate the hypothesis that mortality induced by short term heat stress prior to

winter is the primary factor determining the southern range limit of *P. canadensis.* As regional changes in temperature are not only likely to differ in high temperature spikes, we also tested the impact of other forms of heat stress that are likely to be encountered by *P. canadensis* as climate patterns change.

In particular, global climate change is likely to create shorter winters in temperate regions, where winter temperatures are increasing at a higher rate than summer temperatures (Walther *et al.*, 2002; Schwartz *et al.*, 2006). Therefore, pupae will not only be exposed to higher incidences of temperature spikes prior to winter, but also to prolonged periods at or near metabolic thresholds (longer springs and autumns). Here we evaluate the effects of prolonged warm temperatures prior to the onset of winter (long autumn) on *P. canadensis* pupae and the effects of prolonged periods at temperatures in between winter conditions and those generally experienced prior to emergence (long spring) on pupae of *P. canadensis* and *P. glaucus.*

Materials and Methods

Insects

Pupae were obtained by mass rearing larvae on *Prunus serotina* branches from eggs laid by wild-caught females in 2005 and 2006. *P. canadensis* females were collected from the first flight in the Battenkill River Valley area at the New York/Vermont border U.S.A (43.2° N latitude), and *P. glaucus* females were collected in Lancaster Co. in south-eastern Pennsylvania, U.S.A (40° N latitude).

Experiment 1: Short Term Heat Stress Simulations

On 27 September, 2005 pupae of *P. canadensis* (n= 240) and *P. glaucus* (n=240) were randomly divided into three groups containing 80 pupae of each species. The first group was kept for 14 days in a chamber maintained at 12 hrs of light at 37°C and 12 hrs of dark at 22°C, the second group was kept in the same chamber for 7 days and then placed in a chamber set at 22°C (12:12 hrs Light:Dark) for 7 days, and the third group was placed for 14 days in the chamber maintained at 22°C (12:12 hrs Light:Dark). After this period all pupae were kept for 3 days at 22°C (12:12 L:D), followed by 3 days at 14°C (18:6 L:D), and subsequently maintained in the dark at 3-4°C throughout the remainder of the winter.

On 24 April 2006 pupae were removed from winter storage and placed at 14°C (18:6 L:D) for three days and then equally separated into four chambers. Two chambers were set to "warm" emergence conditions set at18 hrs of light at 24°C and 6 hrs of dark at 20°C (avg. = 22°C), and two chambers set to "cool" emergence conditions at 18 hrs of light at 16°C and 6 hrs of dark at 10°C (avg. = 14°C). Emergence conditions were selected to represent approximate average temperatures encountered during early spring ("cool") and late spring ("warm") in areas near the southern edge of the hybrid zone.

Measurements taken. On September 26 & 27, 2005 and on April 23 & 24, 2006 all pupae were weighed and their length was recorded using a slide calliper. Pupal weight loss was recorded as the weight prior to treatments minus the pupal weight after overwintering. Pupal condition (used as a covariate) was estimated separately for *P. canadensis* and *P. glaucus* as the residuals of a regression of weight on length (*P.*

canadensis $r^2 = 0.87$, *P. glaucus* $r^2 = 0.83$). In the spring of 2006 the emergence success and condition of the wings of the emerging adults was recorded. The condition was recorded as not crumpled (=normal), ¼ crumpled, ½ crumpled, ¾ crumpled, and completely crumpled. Pupae that were dead and butterflies that were unable to fully emerge or unable to fly (wings 3/4 or completely crumpled) were considered dead.

As butterflies emerged they were allowed to dry at which time they were placed in envelopes and kept in a refrigerator at approximately 6°C for 4-8 hours and then their weights, wing length, and abdomen size were recorded. Abdomen size was recorded by measuring the abdomen length (*l*), width (*w*), and thickness (*t*) with a slide calliper and calculating the size as an ellipsoid $V=\pi lwt/6$. Percent weight lost was calculated as (initial pupal weight - emergence weight)/initial pupal weight.

Experiment 2: Prolonged Summer Simulations

From 19 August, 2005 to 19 September, 2005 pupae of *P. canadensis* were placed in chambers maintained at a photoperiod of 18hrs light and 6 hrs dark and temperatures of either 18°C (n= 84), 22°C (n= 146), or 26°C (n= 96). On 19 September, 2005 half the pupae at each temperature were removed and placed at 14°C for 3 days and subsequently kept in the dark at 3-4°C throughout the winter. The remaining half was kept for an additional 30 days at their respective temperatures to simulate a prolonged summer and then slowly cooled to simulate a progressive drop in temperature. Pupae were slowly cooled by sequentially placing pupae for two weeks in chambers set at 4°C lower than the previous chamber until they were placed at 14°C, and subsequently kept in the dark at 3-4°C throughout the winter. Therefore pupae starting at 26°C were placed for two weeks

at 22°C, 18°C, 14°C, and finally at 3-4°C for the winter; while pupae starting at 18°C were only placed at 14°C for two weeks and then placed at 3-4°C for the winter.

On 2 May 2006 pupae were placed at 14°C (18:6 L:D) for three days and then equally separated into four chambers for spring emergence of adults. As in the previous experiment pupae were placed in two chambers set to "cool" emergence conditions at 14°C (18:6 L:D), and two chambers set to "warm" emergence conditions at 22°C (18:6 L:D). As in the previous experiment emergence conditions were selected to represent approximate average temperatures encountered during early spring ("cool") and late spring ("warm") in areas near the southern edge of the hybrid zone.

Measurements taken. On 19 & 20 September 2005, and again on 1 & 2 May 2006 all pupae were weighed and their length measured and pupal condition estimated as described in as described in Experiment 1. As butterflies emerged their condition, weight, and wing length were recorded as in Experiment 1.

Experiment 3: Warm Winter Simulations

In these studies we considered "cold" winter temperatures to be our standard winter temperature (3-4°C), and "cool" winter temperatures to be temperatures between our standard winter temperature and 10°C. We chose 10°C as an upper limit due to the very slow development of *P. glaucus* pupae at temperatures near 10°C [e.g. average emergence time for *P. glaucus* pupae at 11°C is over 130 days for males and 280 days for females (Scriber unpublished results)].

We first tested the impact of prolonged "cool", rather than "cold" winter temperatures on the survival of *P. canadensis* and *P. glaucus* pupae in 2006. On 2 May 2006 pupae of *P. canadensis* and *P. glaucus* kept at the same autumn and winter conditions as controls in the short term heat stress simulations were placed at 6-8°C in the spring of 2006 for 14 (n=40 pupae), or 42 (n=110 pupae) days. They were then placed at 14°C (18:6 L:D) for three days, and subsequently at 22°C (18:6 L:D) for adult emergence. The emergence success and condition of the wings of the emerging adults was recorded as above. The pupae used in this experiment had undergone a complete overwintering period (Mid-October to April/May) under standard conditions and may have been more sensitive than pupae only experiencing a warmer winter.

In 2007 we re-evaluated the response of *P. canadensis* and *P. glaucus* pupae to "cool" winter conditions. However, in this experiment we used pupae that had not yet experienced a complete overwintering period. On 1 February, 2007 overwintering pupae (maintained at 3-4°C) of *P. canadensis* and *P. glaucus* were weighed and separated into three group each composed of 60 *P. canadensis* (n= 180) and 30 *P. glaucus* (n=90). The first group was maintained at 3-4°C as a control, the second group was placed in a chamber set at 10°C 12:12 L:D, and the second group was placed in a chamber maintained at 12 hrs of light at 10°C and 12 hrs of dark at 6°C. On April 13 2007 all pupae were weighed and placed at 10°C 12:12 L:D for 1 day, then at 14°C 12:12 L:D 2 days, and finally at 22°C 18:6 L:D until they emerged. On August 25 2007 all remaining dead pupae were removed and sexed.

Table 2.1. Summary of temperature conditions applied in the three experiments presented.

	Autumn	Winter	Spring
Short Term Heat Stress Simulations	0, 1, or 2 weeks at high temperature conditions and then set for overwintering at 4°C.	All pupae maintained at 4°C.	Pupae set at warm or cool emergence conditions.
Prolonged Summer Temperature Simulations	Pupae maintained until the end of summer (1 month exposure) or into autumn (2 month exposure) at 18, 22, or 26 °C. Then set for overwintering at 4°C.	All pupae maintained at 4°C.	Pupae set at warm or cool emergence conditions.
Warm Winter Simulations	All pupae maintained at 18 °C, until early-mid autumn and set for overwintering at 4°C.	Pupae placed from mid-winter to early spring at either 4°C (control), alternating temperatures 10:6°C (12hr:12hr), or 10°C constant.	All pupae set at warm emergence conditions.

Statistical Analyses

Survival of *P. canadensis* pupae in the short term heat stress simulations, prolonged summer simulations, and warm winter simulations were analysed as Analyses of Deviance by applying a GLM (Family = Binomial, logit link) using R (R Development Core Team 2006). Due to exceptionally high survival of *P. glaucus* Analyses of Deviance were not possible as assumptions of dispersion were violated. Therefore survival of *P. glaucus* in all experiments was analysed as Binomial tests.

Wing length, abdomen size and percent weight loss for butterflies that emerged with wings less than ¼ crumpled in the short term heat stress simulations were analysed separately for *P. canadensis* and *P. glaucus* using ANCOVAs with autumn and spring temperature conditions (and their interaction) and sex as fixed factors and initial pupal condition and initial weight as covariates. As all ANCOVAs included the same factors and covariates for these analysis estimates of partial omega square (ω^2) are comparable between these analyses. ω^2 is a measure of effect size ranging from 0 to 1 (as long as the F-ratio is greater than 1), for which the larger the value the larger the effect of the treatment on the measured variable.

Pupal weight loss and emergence times for each species in the warm winter simulation experiment were analysed as separate ANCOVAs, using initial weight as a covariate and temperature treatment and sex as fixed factors. All ANCOVAs and ω^2 estimates were based on Type "III" sums of square using the CAR package available for R (Fox, 2006). In all analyses performed no significant differences were found between replicate chambers, therefore it was dropped as a factor in the analyses.

Results

Experiment 1: Short Term Heat Stress Simulations

The results from this study indicate that while unusually high autumn temperatures did have a significant impact upon *P. canadensis* survival (Table 2.2 and Fig. 2.1); it was much lower than the near complete mortality reported by Scriber *et al.*(2002) for similar temperatures imposed on pupae during emergence in spring. Results from Scriber *et al.*(2002) indicated that even 4 days at 36 °C during spring were essentially lethal for *P. canadensis* pupae and also induced a high mortality on *P. glaucus* pupae.

Table 2.3 provides a summary of the ANCOVAs performed separately for *P. canadensis* and *P. glaucus* for the percent weight loss from pupae to emergence, wing lengths, and abdomen size. These measurements provide a method to assess the stress induced by autumn and spring temperature treatments on the two species. An ANCOVA of pupal weight loss of *P. glaucus* pupae indicated a significant effect of high autumn temperature ($F_{2,259}$ P= 0.002). However, the effect size was fairly small ω^2 =0.036 and survival of *P. glaucus* was excellent under all treatment conditions (Fig. 2.1). In contrast *P. canadensis* survival was significantly lowered by both heat stress in autumn and cool spring emergence conditions (Fig. 2.1 and Table 2.2). As with *P. glaucus* an ANCOVA of pupal weight loss of *P. canadensis* for pupae in the short term heat stress simulation was significant ($F_{2,253}$ P< 0.001), but the effect size was considerably larger ω^2 =0.3 (larger effect size = greater treatment effect).

Table 2.2. The effect of spring emergence temperatures, high temperature spikes in autumn, initial weight, and initial body condition on the successful emergence (survival) of *P. canadensis* pupae. Analysis of Deviance performed by applying a GLM (Family = Binomial, logit link) using R (R Development Core Team 2006).

Terms added sequentially	*df*	Deviance	Residual *df*	Residual deviance	P(χ^2)
NULL			236	265.996	
Spring Temp.	1	3.986	235	262.01	0.046
Autumn Temp.	2	12.871	233	249.139	0.02
Initial weight	1	9.022	232	240.117	0.003
Initial Body Condition	1	8.752	231	231.365	0.003
Spring temp*Autumn Temp	2	8.341	229	223.024	0.015

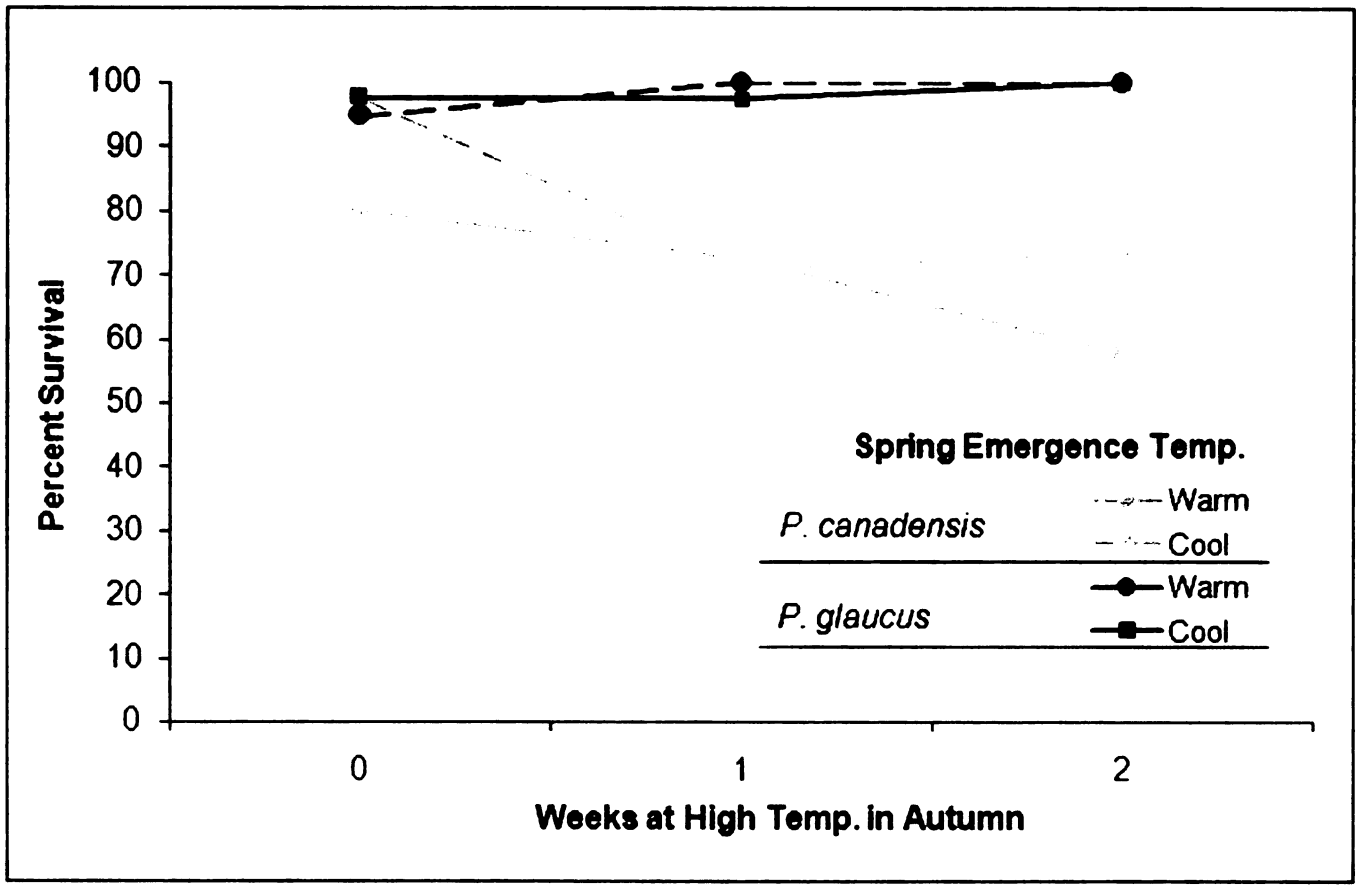


Figure 2.1. Percent survival of *P. glaucus* and *P. canadensis* pupae placed in environments simulating heat waves in early Autumn (37°C days and 22°C nights for 0, 1, or 2 weeks) and emerged in environments simulating long springs, "cool" spring emergence conditions (16°C days and 10°C nights) or short springs, "warm" spring emergence conditions (24°C days and 20°C nights). Dark lines represent *P. glaucus* and gray lines *P. canadensis.* For each species solid lines represent pupae emerged in chambers set at cool emergence conditions, and dashed lines pupae emerged in chambers at warm emergence conditions

Table 2.3. Summary of results for measurements taken on pupae and butterflies of *P. glaucus* and *P. canadensis* from Experiment 1: Short Term Heat Stress Simulations. Values in the measurement column represent the mean ± SE for males and females. P-values and ω^2s in the autumn and spring treatment effect columns are derived from ANCOVAs using Type "III" sums of square including sex as an independent variable and initial body weight and initial body condition as covariates in R (R Development Core Team 2006).

Species	Measurement		Autumn Treatment Effect		Spring Treatment Effect	
	Percent Weight Loss (Pupae to Emergence)					
P. glaucus	♂: 55.1 ± 0.5 %	♀: 45.6 ± 0.5 %	P=0.383	ω^2 = NA	P=0.98	ω^2 = NA
P. canadensis	♂: 52.5 ± 0.6 %	♀: 45.8 ± 0.5 %	P<0.001	ω^2 = 0.05	P=0.01	ω^2 = 0.02
	Relative Abdomen Size					
P. glaucus	♂: 244.1 ± 6 mm^3	♀: 346.9 ± 8 mm^3	P=0.897	ω^2 = NA	P=0.001	ω^2 = 0.02
P. canadensis	♂: 169.7 ± 5 mm^3	♀: 236.1 ± 6 mm^3	P=0.064	ω^2 = NA	P=0.072	ω^2 = NA
	Wing Length					
P. glaucus	♂: 49.8 ± 0.3 mm	♀: 52.2 ± 0.3 mm	P=0.059	ω^2 = NA	P<0.001	ω^2 = 0.11
P. canadensis	♂: 44.2 ± 0.3 mm	♀: 46.1 ± 0.2 mm	P=0.014	ω^2 = 0.01	P<0.001	ω^2 = 0.039

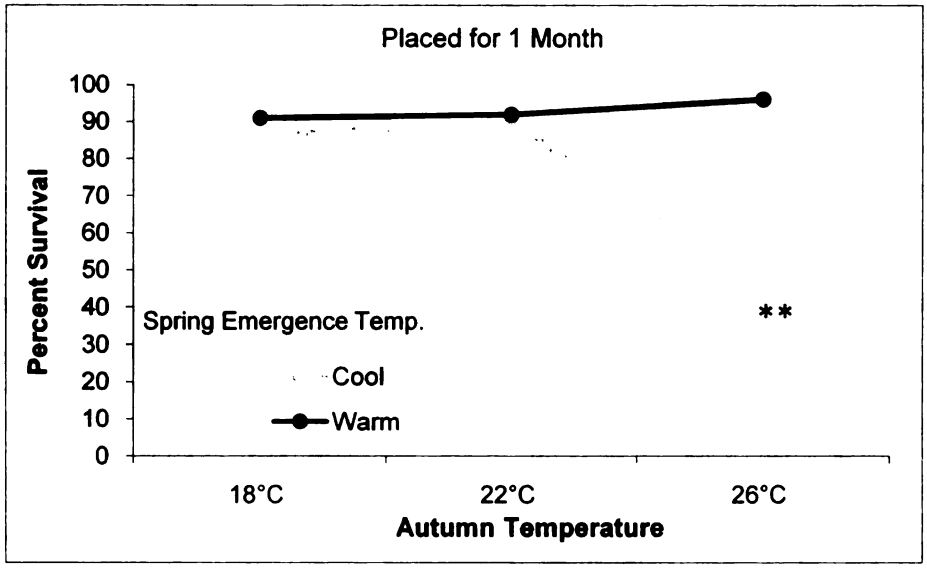
An interesting result observed was that when we compare the effect size (ω^2) for spring emergence conditions on wing length and abdomen size we see a greater treatment effect of emergence conditions for *P. glaucus* than *P. canadensis* (Table 2.3). This difference in the reaction to emergence conditions may be a reflection of the larger reserves present in *P. glaucus.* It is likely that *P. glaucus* is capable of buffering the effects of longer (cooler) spring emergence conditions by reallocating resources, as has been seen in other insects in response to various forms of stress (e.g. Angelo and Slansky 1984, Boggs and Freeman 2005, Jannot *et al.*2007).

The overall results from this experiment indicate a difference in tolerance to heat stress between pupae of *P. canadensis* and *P. glaucus* prior to winter (prior to imago development), but far less severe than observed during emergence in spring (Scriber *et al.*2002) and insufficient for rare heat waves to set a southern range limit for *P. canadensis*.

Experiment 2: Prolonged Summer Simulations

In this experiment we found that extending the exposure to warm temperatures did significantly increase the mortality rate of *P. canadensis.* An Analysis of Deviance indicated survival was significantly reduced by higher autumn temperatures (deviance = 51.57, d.f. = 2, $P(\chi^2) < 0.001$), prolonged higher autumn temperatures (deviance = 8.99, d.f. = 1, $P(\chi^2) = 0.003$), lower spring temperatures (deviance = 13.55, d.f. = 1, $P(\chi^2) < 0.001$), and a significant interaction between spring and autumn temperatures (deviance = 9.21, d.f. = 2, $P(\chi^2) = 0.01$). More detailed observations of the results from this experiment are summarised in Figure 2.2. In Figure 2.2a we can see the survival of *P. canadensis* pupae kept for 31 days (August 19-September 19) at

(a)



(b)

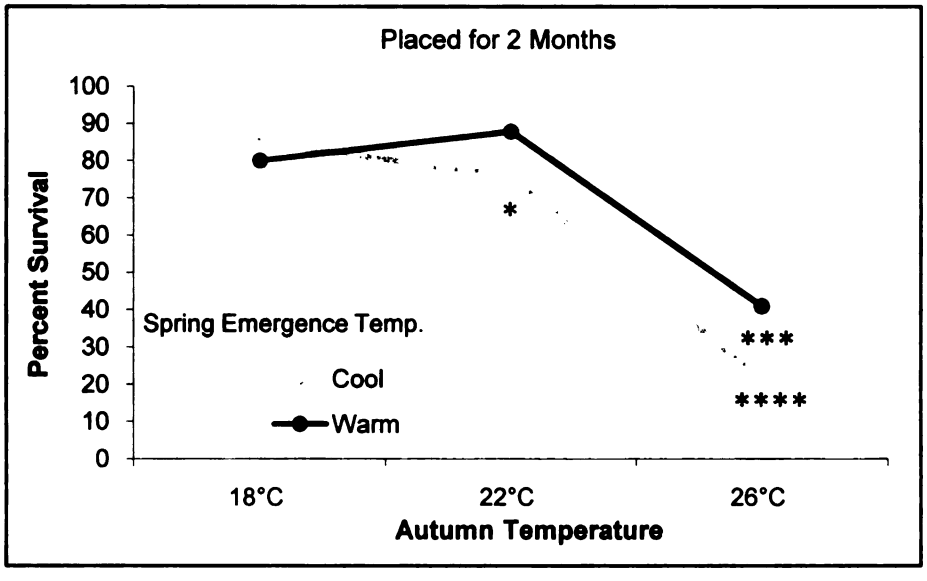


Figure 2.2. Percent survival of *P. canadensis* pupae maintained at 18°C, 22°C, or 26°C (a) for one month or (b) two months during autumn. Pupae were subsequently emerged in "cool" spring emergence conditions (14°C) or "warm" spring emergence conditions (22°C). Survival significantly lower than expected values based on binomial tests are represented by * for 90%, ** for 60%, *** for 50%, and **** for 40%.

temperatures likely to be observed during this period (18°C and 22°C) had excellent survival. Only pupae exposed to both 26°C and cool emergence conditions (14°C) experienced a significant decrease in survival. This indicates that exposure to 26°C in autumn significantly stressed the pupae of *P. canadensis.* This effect became more evident when pupae were maintained for 61 days (August 19- October 19) at 18°C, 22°C, or 26°C (Fig. 2.2b), as is evidenced by significantly lower survival under both emergence conditions.

The results from this experiment indicate that prolonged exposure to warm temperatures can significantly stress the pupae of *P. canadensis.*

Experiment 3: Warm Winter Simulations

Successful emergence of *P. glaucus* placed for 14 or 42 days at 6-8 °C prior to being set for emergence in 2006 was high (95% and 93.5% respectively) and not surprisingly binomial tests indicated they were not significantly lower than 95% (P=0.64). For *P. canadensis* successful emergence of pupae left for 14 days at 6-8 °C was lower, but not unusual (80%). A binomial test did not indicate a significant difference than the survival of the controls (90%) (P= 0.13). However, the number of successful emergences of *P. canadensis* placed at 6-8 °C for 42 days prior to emergence was very low, 42%, and significantly lower than 60% (P=0.005).

The emergence of pupae placed at 10 °C or temperatures alternating between 10 °C and 6 °C from 1 February, 2007 to 13 April, 2007 indicated a very similar pattern to those placed at 6-8 °C for 42 days in 2006. In particular we found no significant difference in emergence success for *P. glaucus* (all treatments had emergence success >

95%) and a significantly lower survival for *P. canadensis* in our treatments relative to our control (deviance = 29.5, d.f. = 2, $P(\chi^2) < 0.001$) (Fig. 2.3). In addition, we found a significantly greater percent weight loss in *P. canadensis* pupae placed at 10 °C and temperatures alternating between 10 °C and 6 °C relative to the control ($F_{2,174} = 61.8$, $P< 0.001$; Fig. 2.4), and no significant difference in percent weight loss between *P. glaucus* pupae in different temperature regimes ($F_{2,80} = 2.0$, $P=0.15$; Fig. 2.4).

Finally, when pupae were placed at 22 °C for emergence in 2007 an ANCOVA indicated significantly different emergence time for *P. canadensis* pupae placed at 10 °C (9.4 ± 0.4 days), temperatures alternating between 10 °C and 6 °C (11.8 ± 0.3 days), and the 4 °C control (16.0 ± 0.4 days) ($F_{2,113} = 81.52$, $P< 0.001$). Tukeys HSD indicated significant differences between the three temperature conditions at $\alpha=0.05$. For *P. glaucus*, we also found a significant difference in emergence times between pupae placed at the three temperature regimes ($F_{2,113} = 14.0$, $P< 0.001$). However, Tukeys HSD indicated that differences were only significant between pupae placed at 10 °C (15.2 ± 0.85 days) and the other two treatments; temperatures alternating between 10 °C and 6 °C (19.5 ± 0.57 days) and the control (19.7 ± 0.63 days). In addition, the effect size (ω^2) for the treatment effect on *P. canadensis* is 0.56 while that for *P. glaucus* is only 0.24, indicating a much stronger effect of temperature treatment on the emergence time of *P. canadensis* than *P. glaucus.*

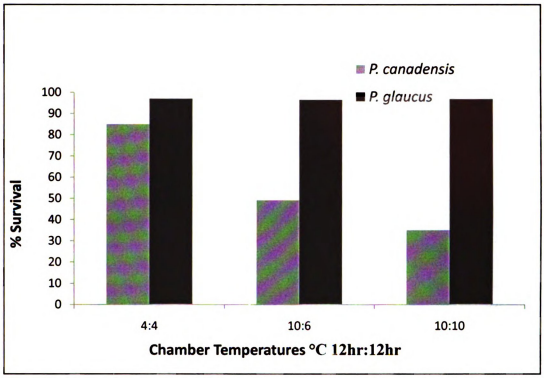


Figure 2.3. Percent survival of *P. glaucus* and *P. canadensis* pupae maintained in chambers set at continuous 4°C (4:4), alternating 12 hours at 10°C and 12 hours at 6°C (10:6), or continuous 10°C (10:10) from midwinter to early spring.

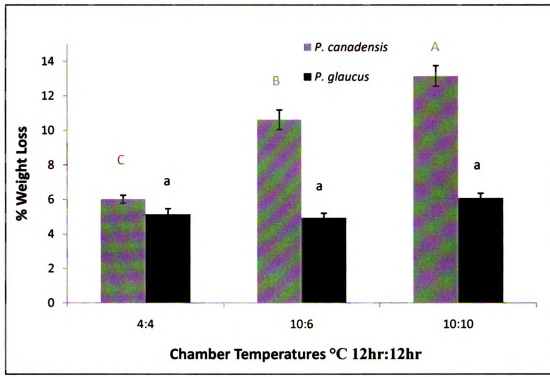


Figure 2.4. Percent weight loss of *P. glaucus* and *P. canadensis* pupae maintained in chambers set at continuous 4°C (4), alternating 12 hours at 10°C and 12 hours at 6°C (10:6), or continuous 10°C (10) from midwinter to early spring. Within each species means followed by the same letter are not significantly different at the $p<0.05$ level (Tukeys HSD).

Discussion

Our results indicate that unusually hot late summer/autumn temperatures are not the primary cause of the southern range limit of *P. canadensis*. While pupae of *P. canadensis* exposed to short periods of unusually high autumn temperatures did have a significantly lower survival (Fig. 2.1), it was much lower than the near total mortality for high spring temperatures reported by Scriber *et al.*(2002). Extreme responses, such as the near complete mortality of *P. canadensis* exposed to 4 days at 36 °C during spring (Scriber *et al.*, 2002), are likely to be sufficient for rare events to pose a significant selective pressure. However, our results did not indicate such an extreme response for *P. canadensis* (Fig. 2.1). The temperatures we exposed pupae to (37 °C days and 22 °C nights) are extreme, and although possible, these temperatures are rare and unlikely to be present for prolonged periods of time (e.g. 1-2 weeks). The discrepancy between this study and the one presented by Scriber *et al.*(2002) lies in the physiological state of the pupae prior to overwintering and after overwintering. Prior to overwintering pupae are in a quiescent phase more likely to tolerate temperature extremes, while during spring, diapause is broken and formation of the imago inside the pupae begins, and sensitivity during this stage is likely a representation of temperature thresholds during imago development. Temperature averages exceeding 30 °C may occur in the summer, but are highly unlikely to occur in the spring when the imago is developing. Therefore it is unlikely that spring thermal stress is the primary cause of the Southern range limit of *P. canadensis*.

We did find a greater tolerance to high temperatures in *P. glaucus* than *P. canadensis* that is likely to be a reflection of differences in metabolic thresholds and/or

energy reserves in the two species. In contrast to *P. canadensis, P. glaucus* did not experience a significant decrease in survival rate or weight due to our short term heat stress simulations (Fig. 2.1 and Table 2.3). In addition, cool emergence temperatures did not significantly impact *P. glaucus* survival and weight, while it did have a significant effect upon *P. canadensis* (Fig. 2.1 and Table 2.3). Metabolism during diapause is significantly reduced, but once diapause is broken, metabolism rates become higher and prolonged periods of development are likely to significantly reduce energy reserves. Pupae placed for emergence in 2006 at cool emergence temperatures (14 °C average) took approximately twice as long to emerge as those placed at warmer emergence temperatures (22 °C average). This extended period of development significantly reduced the survival of *P. canadensis,* while that of the larger *P. glaucus* was not affected (Fig. 2.1). This result may be, at least in part, due to the fact that in general, smaller individuals are believed to have fewer metabolic resources and have higher metabolic rates (Peters, 1983; Gillooly *et al.,* 2001).

Although, *P. glaucus* did not have a reduction in survival, spring emergence temperature did cause a significant reduction in *P. glaucus* abdomen size and a stronger negative effect on the wing length of emerging adults (Table 2.3). These differences are indicative of larger reserves in *P. glaucus* dampening the negative effects of prolonged developmental time on survival. In addition, although there were significant reductions in abdomen size and wing length due to emergence conditions, there was no significant effect of emergence conditions on *P. glaucus* overall weight (Table 2.3). This result is a strong indication that *P. glaucus* is capable of buffering the effects of longer (cooler) spring emergence conditions by reallocating resources, as has been seen in other insects

in response to various forms of stress (e.g. Angelo and Slansky 1984, Boggs and Freeman 2005, Jannot *et al.*2007).

In addition to potentially higher metabolic reserves being present in *P. glaucus,* it is also apparent that *P. canadensis* has a lower metabolic threshold. In our warm winter simulations *P. canadensis* weight loss was significantly higher relative to the controls, while that of *P. glaucus* was not significantly different (Fig. 2.4). In addition, emergence times were significantly shorter for *P. canadensis* exposed to warm winters than for *P. glaucus* exposed to warm winters (see results). Together these results indicate that *P. canadensis* had lower developmental thresholds in our warm winter simulations than *P. glaucus*, which is likely to be the cause of the high mortality rates observed (Fig. 2.3). The ability to have a faster imago development is likely adaptive in the majority of the *P. canadensis* range as the available growing season is constrained, but in more southern latitudes it may pose a significant cost.

The prolonged summer simulations indicated that *P. canadensis* is also sensitive to prolonged periods at relatively mild warm temperatures prior to winter conditions (Fig. 2.2). The survival rate of individuals exposed to one or two months of warm conditions was far lower than those exposed to short periods of high heat (Figs. 2.1 and 2.2). Although diapausing pupae have lowered metabolic rates, some metabolism is still present and high temperatures can be metabolically costly (Hahn & Delinger, 2007). The low survival rates of *P. canadensis* exposed to prolonged periods of warm temperature or slightly above overwintering conditions (Figs. 2.2 and 2.3) indicate that mild but prolonged shifts in temperature may be a more significant stress than occasional short heat waves prior to overwintering.

Although short periods of unusually hot summers do not appear sufficient to act as the primary limit to the southern distribution of *P. canadensis,* higher temperatures do appear to pose a significant cost to *P. canadensis* that could limit its range. However, the effects of temperature appear to be gradual (e.g. increased warm temperature conditions) and we would not expect a sharp southern boundary to the range as is observed (Scriber *et al.,* 2003). It is likely that the sharp boundary observed for *P. canadensis* is due to a combination of environmental factors that shift at the ecotone between boreal and temperate deciduous forests, one of these being the temperature regime.

Other potential factors associated with the sharp boundary include the shift in host plants available and the presence of *P. glaucus.* At this ecotone the most common host of *P. canadensis, Populus tremuloides* (Salicaceae), begins to decline and the preferred host of *P. glaucus, Liriodendron tulipifera* (Magnoliaceae), becomes more prevalent. While *L. tulipifera* is toxic to *P. canadensis* larvae female *P. canadensis* will readily oviposit on it. The presence of *P. glaucus* is also likely to affect that of *P. canadensis* through factors such as apparent competition (e.g. through common predators) and/or significantly reducing successful matings. Both *P. canadensis* and *P. glaucus* males strongly prefer mating with *P. glaucus* females (Deering & Scriber, 2002). Although *P. glaucus* and *P. canadensis* crosses produce fertile offspring (e.g. Scriber *et al.,* 1995; Scriber *et al.,* 2003), the female offspring from these crosses emerge 2-3 weeks after the flight period of *P. canadensis* and *P. glaucus* males (Scriber & Ording, 2005; Scriber *et al.,* 2007), significantly reducing the likelihood of mating for such hybrid females.

An indication that the southern range limit for *P. canadensis* may not be primarily maintained by abiotic factors alone is that prior to 1998, *P. canadensis*-like traits were

observed farther south into *P. glaucus* populations than *P. glaucus* traits were into *P. canadensis* populations (Scriber, 1990). As the *P. glaucus* northern range limit is defined by the thermal environment that allows the completion of two generations (Scriber, 1994), an environmental barrier to movement was present for *P. glaucus.* In contrast, such a sharp environmental barrier may not have been present for *P. canadensis*; instead a combination of factors (including temperature, host availability, and the presence of *P. glaucus*) may have all acted to create the southern range limit of *P. canadensis.* However, since 1998 the rapid increases in temperature have changed the previous pattern by increasing the geographic area where *P. glaucus* can complete 2 generations (Scriber & Ording, 2005; Scriber *et al.,* 2007). This environmental change has lead to the introgression of *P. glaucus* traits into *P. canadensis* populations including the formation of a late emerging hybrid swarm population (Scriber, 2002; Scriber & Ording, 2005; Scriber *et al.,* 2007).

Conclusion

Occasional very hot summer temperatures do not appear to be the primary cause of the southern range limit of *P. canadensis*. However, *P. canadensis* does exhibit a considerably lower tolerance to high temperature extremes and conditions simulating shorter/warmer winters than *P. glaucus.* These results suggest that differential temperature tolerance may be a factor in maintaining the parapatric species borders in this hybrid zone. In particular the results suggest that the expenditure of metabolic reserves is likely to be an important component of the southern range limit of *P. canadensis.* As the climate warms, cold temperatures are expected to be shorter, in

particular transitions between cold temperatures to hot temperatures are expected to be longer and arrive earlier (i.e. shorter winters and longer springs) (Schwartz *et al.*, 2006). Therefore it is likely that climate change will greatly increase the likelihood of local extinctions of cold adapted insects that diapause as pupae, even if they can tolerate temperature spikes and don't emerge out of synchrony with their hosts.

CHAPTER 3

Mercader RJ, Scriber JM (2007) Diversification of host use in two polyphagous butterflies: Differences in oviposition specificity or host rank hierarchy? Entomologia Experimentalis et Applicata 125: 89-101.

DIVERSIFICATION OF HOST USE IN TWO POLYPHAGOUS BUTTERFLIES: DIFFERENCES IN OVIPOSITION SPECIFICITY OR HOST RANK HIERARCHY?

Abstract

Novel host use may represent an initial step towards diversification or radiation onto novel hosts within an evolutionary lineage, particularly if a shift in host plant preference ranking takes place. Polyphagous stages of evolutionary lineages may represent transitional states in which novel host associations are more likely to develop, but may be more difficult to detect experimentally. The polyphagous sister species *Papilio glaucus* L. and *Papilio canadensis* (Lepidoptera: Papilionidae; these *Papilio* = *Pterourus*) are known to exhibit differences in host plant use, despite significant overlap in host use abilities, providing an opportunity to examine how host shifts in polyphagous species may occur and what the implications for future divergence may be. In particular, we were interested in 1) determining whether differences in oviposition behavior of these species were due to changes in specificity or shifts in host plant hierarchy, 2) whether the varying preference for primary hosts also affected the preference for secondary hosts, and 3) what the oviposition preferences of a new hybrid swarm population are. We examined more than 40,000 oviposition bouts from more than 400 *P. glaucus, P. canadensis,* and hybrid females placed in seven, three, or two choice assays. In each of the choice assays, leaves from plants in different plant families of varying suitability for *P. glaucus* and *P. canadensis* larvae were used. We found the primary difference between *P. glaucus* and *P. canadensis* to be limited to a Z-linked shift in host rank hierarchy due to an acceptance of

Populus tremuloides Michx. (Salicaceae) and reduced specificity for *Liriodendron tulipifera* L. (Magnoliaceae) in *P. canadensis.* In addition, we found the absence of the Z-linked oviposition acceptance of *P. tremuloides* in a recently formed allochronically separated hybrid swarm population found in *P. canadensis* territory at the northern border of the *P. glaucus* and *P. canadensis* hybrid zone.

Introduction

The divergence of insect species through host plant specialization has been proposed as a mechanism to explain the great diversity of plant feeding insects, but specialization alone would be a dead end process. Janz *et al.*(2006) proposed a scenario of oscillating host expansion and specialization to help resolve this apparent dilemma. Within this scenario, polyphagy is a transitional state that allows the incorporation of new hosts. It thus prevents specialization from reaching a dead end and may even facilitate radical host shifts (Janz *et al.*, 2001; Weingartner *et al.*, 2006). In addition, colonization of new hosts leading to radiation, rather than co-cladogenesis, has been gaining support as the reason for the association between plant feeding insects and their hosts (Janz *et al.*, 2001; Nosil, 2002; Percy *et al.*, 2004; Braby & Trueman, 2006; Lopez- Vaamonde *et al.*, 2006; and Murphy & Feeny, 2006). Therefore, host shifts in polyphagous insects are of particular interest, as they may represent the initial steps towards diversification or the acquisition of novel hosts within an evolutionary lineage.

Perhaps due to the greater abundance of specialized insects and an easier detection of host shifts within them, the majority of studies on host shifts have concentrated on monophagous or oligophagous species (e.g., Carroll & Boyd, 1992;

Radtkey & Singer, 1995; Menken, 1996; Feder *et al.*, 1998; Abrahamson *et al.*, 2001; Hora *et al.*, 2005; Ohshima & Yoshizawa, 2006; Stastny *et al.*, 2006; but see Thompson, 1998; Janz *et al.*, 2001 for shifts between specialized and generalized forms).The closely related sibling species *Papilio glaucus* L. and *Papilio canadensis* R & J (Lepidoptera: Papilionidae; these *Papilio* = *Pterourus*) offer the unusual opportunity to examine a shift in host use between two polyphagous forms. These sibling species exhibit the highest degree of polyphagy amongst the 560+ species of Papilionidae (Scriber, 1984), as together their larvae can consume 30-40 species in 14 plant families (Scriber, 1988). Despite their high level of polyphagy and a significant host overlap, both species exhibit differences in suitability of some of their primary hosts for neonate larvae (Scriber, 1996a). In particular, the most common host plant family of *P. canadensis*, Salicaceae, is toxic to *P. glaucus* larvae, while the preferred host of *P. glaucus*, tulip tree, *Liriodendron tulipifera* L. (Magnoliaceae), is toxic to *P. canadensis* larvae.

For plant feeding insects with larvae that develop on a single host, such as tree feeding *Papilio*, changes in oviposition behavior of adults are necessarily required for host range shifts. Behavioral adaptations to use new hosts have been proposed to precede physiological adapations (Futuyma *et al.*, 1984; Janz *et al.*, 1994) and to be evolutionarily labile (Wasserman & Futuyma, 1981; Gassman *et al.*, 2006). In addition, the common observance of oviposition "mistakes" (Straatman, 1962; Wiklund, 1975; Chew, 1977; Berenbaum, 1981; Scriber *et al.*, 1991; Scriber, 1993; Larsson & Ekbom, 1995; Renwick, 2002; Graves & Shapiro, 2003), lack of concordance between preference and performance in many organisms (Thompson & Pellmyr, 1991; Mayhew, 1997), and variation in oviposition profiles due to thermal constraints (e.g., Scriber & Lederhouse,

1992; Scriber, 1996b, 2002a) support the notion that the apparently close associations between plant feeding insects and their host plants are actually rather flexible.

Shifts in oviposition behavior may be due to changes in specificity in which the proportion of eggs laid on lower ranking hosts differs, or due to shifts in the host preference ranking (Courtney *et al.*, 1989). Changes in specificity are likely to lead to shifts between specialist and generalist forms which may lead to diversification within the associated plant lineage. However, changes in specificity are unlikely to significantly alter host plant associations, because the primary host associations remain. In contrast, shifts in host preference rank hierarchy can lead to shifts away from associated plant lineages towards the plant lineage of secondary hosts, altering primary host plant associations within an insect lineage. In some instances, shifts in host preference rank hierarchy may also include the acceptance of a novel host or plant family unused within the evolutionary lineage of the organism. Such shifts are likely to open an entirely novel niche which could lead to adaptive radiation. However, these shifts may be uncommon as is reflected in the evolutionary conservatism of host plant affiliations in plant feeding insects (Ehrlich & Raven, 1964; Janz *et al.*, 2001; Weiblen *et al.*, 2006).

Within the *P. glaucus* species group there has been significant diversification in associations with plant families used (Scriber, 1996a) and significant differences in oviposition behavior have been noted between the closely related *P. glaucus* and *P. canadensis* (Scriber *et al.*, 1991; Scriber, 1994). Studies comparing the oviposition behavior of *P. glaucus* and *P. canadensis* have primarily used three choice oviposition arenas with leaves of *L. tulipifera* (Magnoliaceae), *Populus tremuloides* Michx. (Salicaceae), and a common host for both species, *Prunus serotina* L. (Rosaceae). These

studies have indicated that in general *P. glaucus* individuals will lay most of their eggs on *L. tulipifera* and very few eggs on *P. tremuloides*, while *P. canadensis* individuals will lay roughly similar quantities of eggs on all three hosts (Figure 3.1).

Despite the intensive work on these species it is still unknown whether the observed differences in oviposition behavior represent differences in specificity or a shift in host plant hierarchy. In addition, it is unknown whether changes in oviposition behavior observed are also found amongst hosts in the other lower ranked plant families used by these polyphagous species. These aspects of their shifts in oviposition behavior are relevant for the potential future divergence within these species or as these two species hybridize.

In recent years gene movement has been seen to increase across a hybrid zone delineating the range limits of *P. glaucus* and *P.canadenis* (Scriber, 2002b). This increase in hybridization has produced an allochronically separated hybrid swarm population (Scriber & Ording, 2005) remarkably similar to a population found in northern Georgia believed to represent a separate species *Papilio appalachiensis* (Pavulaan & Wright, 2002). The larval host use abilities of this delayed hybrid swarm population represent a mixture of the two genomes (Scriber *et al.*, unpublished data) and their oviposition behavior is likely an important indicator of the ecology and the evolutionary potential of this hybrid swarm population if it becomes/remains reproductively isolated.

In order to understand what the difference in oviposition between *P. canadensis* and *P. glaucus* is and what its implications for future divergence are, we assayed *P.*

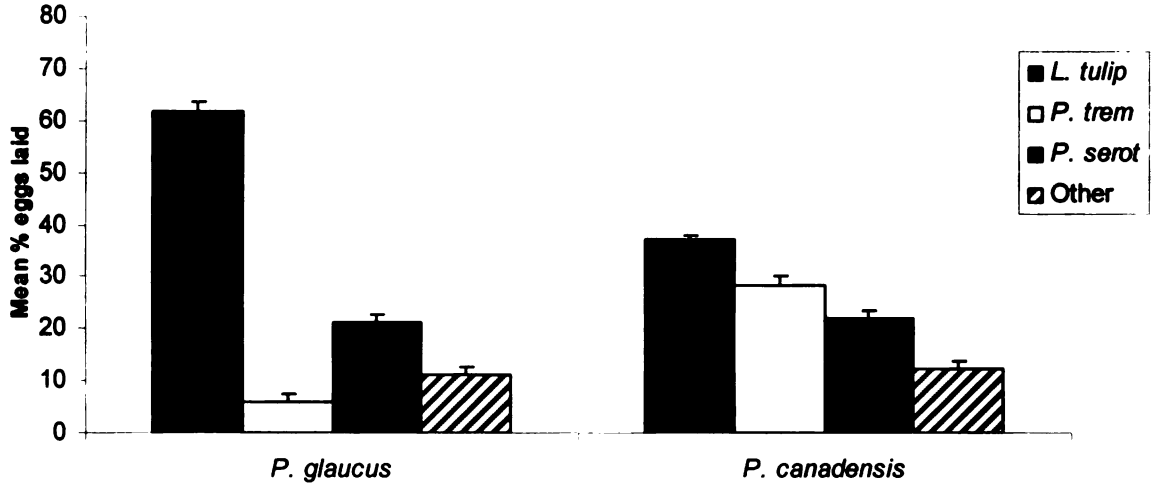


Figure 3.1. Mean percentage + SE of eggs laid by 93 female *Papilio glaucus* from Lancaster Co., PA, and 94 female *Papilio canadensis* from the "early flight" found in the Battenkill River Valley in the New York/ Vermont border. Leaves present in the bioassay were *L. tulipifera*, *P. serotina*, and *P. tremuloides*. The "other" category represents eggs laid on the paper or plastic of the oviposition arena.

canadensis and *P. glaucus* populations near the northern and southern border of the hybrid zone on arenas containing seven, three, or two hosts. We were also able to compare individuals from the newly formed hybrid swarm population in three choice assays to populations of both *P. canadensis* and *P. glaucus*. Using these data we specifically asked the following questions 1) Does *P. canadensis* exhibit a preference for *P. tremuloides* or simply a decreased specificity for *L. tulipifera*?; 2) Does *P. canadensis* exhibit a different oviposition preference than *P. glaucus* for hosts other than *P. tremuloides*?; and 3) What is the oviposition pattern of the newly formed late flight hybrid swarm?

Materials and methods

Insects

Butterflies used in seven choice and two-choice studies in 2006 were reared in 2005 or 2006 from eggs laid by wild-caught females, with the exception of the northern Michigan individuals which were wild-caught individuals. Females of the "early flight" *P. canadensis* were reared on *P. serotina* by either mass rearing in sleeves placed on tree branches or reared independently in a growth chamber at 22 °C in 2005, from eggs of females collected from the first flight (late May-early June) in the Battenkill River Valley area at the New York/Vermont border USA (43° N latitude and 73° W longitude). The *P. glaucus* females from the Pennsylvania population were field-reared in 2005 on *P. serotina* from butterflies collected in Lancaster Co. in south-eastern PA, USA (40° N latitude and 76° W longitude). All females were kept at 3-4 °C throughout the fall and winter of 2005, until they were emerged the spring of 2006. Females from the Georgia

population were field-reared on *P. serotina* in 2006 from females collected in Oglethorpe Co., GA, USA (34° N latitude and 83° W longitude). After eclosion, butterflies were fed a 15-20% honey water solution and stored at 3-4 °C for a maximum of 4 days until they were hand paired and setup in oviposition arenas. Females from the northern Michigan population were field collected in 2006 from Cheboygan and Emmet Counties in MI, USA. (45° N latitude and 84° W longitude). These butterflies were transported in coolers to the MSU laboratory where they were fed a honey water solution and placed in oviposition arenas.

Butterflies used in the three choice assays were a combination of females collected in 2003, 2004, and 2005, and individuals reared on *P. serotina* during 2002, 2003, and 2004. The populations used were the same as those above, with the exception of the "late flight" population. Females from the "late flight" population were derived from wild-caught females from the "false-second" flight in the Battenkill River Valley area at the NY/VT border, USA as described in Scriber & Ording (2005).

Butterflies used in seven choice bioassays in 1993, 1996, and 1998 were a combination of wild-caught and lab reared females. Females were collected in 1993 from Fairbanks, Alaska, USA (65° N latitude), and in 1996 from Menifee Co., Kentucky, (37.9° N latitude) and Lawrence Co., Ohio, USA (38.5° N latitude) and were shipped overnight to our MSU facilities. In 1998, wild-caught *P. canadensis* females from Charlevoix Co., Emmet Co., and Dickinson Co., MI, USA, (45.5-47° N latitude) were transported in coolers to our MSU facilities. All females were fed honey water solution upon arrival to MSU and setup in oviposition arenas. In 1996, four hybrid families were produced by crossing laboratory reared *P. canadensis* females from northern Michigan

with wild-caught males from Menifee Co. KY, USA. The resulting females were then mated and placed in oviposition arenas.

General methods for oviposition assays

The general methods used were similar to those described by Scriber (1993); arenas consisted of clear round plastic containers placed on a rotating platform (approximately 10 revolutions per h) in front of incandescent lights on a L4:D4 h photocycle to maximize egg laying, with leaves of each plant species placed in floral aquapicks. Butterflies were fed a honey-water solution daily and allowed to oviposit until they were too weak to fly (4-12 days). The number of eggs laid on leaves of each plant or non-leaf portions were counted daily and the leaves replaced with new leaves as necessary. No leaf remained for more than 2 days in the assays.

Leaves were collected from trees growing in Ingham County, MI, USA, from locations known to be pesticide free, placed in aquapicks in plastic bags, and placed in a refrigerator for up to 3 days to ensure leaf quality remained high. Leaves used in the assays were collected from at least four trees, with the exception of *L. benzoin* and *B. papyrifera* for which two and one plants were used, respectively, since plants known to be pesticide free were more limited for these two hosts.

***Papilio canadensis* oviposition preference**

Ideally, distinguishing oviposition specificity and host plant rank hierarchy would be accomplished using a combination of sequential no choice trials and simultaneous choice trials. However, the behavior of these butterflies provide a few important experimental

constraints to the use of no-choice trials and interpretations of simultaneous choice trials: 1) the number of eggs laid is highly variable among days and individuals, which requires oviposition data to be analyzed as proportions, and 2) the degree of host specificity exhibited by individual females (even within full sibling families) is highly variable. These limitations prohibit the use of single host arenas and decrease the likelihood of distinguishing between shifts in specificity and host preference rank hierarchy from arenas with multiple hosts, which make direct tests of preference hierarchies difficult. However, we can test for preference hierarchies indirectly by making the assumption that if there is a preference for a particular host then individuals with greater specificity for that host should lay a greater number of eggs on that host and individuals with lower specificity for that host should lay eggs more evenly across hosts. Therefore, given enough choices we should be able to distinguish whether the cause of an increased average proportion of eggs laid on a host are due to the presence of a preference for that host or the reduction in specificity for other hosts.

To test for this relationship, we placed butterflies in oviposition arenas containing leaves from plants in seven different families with varying larval suitability for *P. canadensis* (see above); *L. tulipifera*, *L. benzoin*, *P. tremuloides*, *P. trifoliata*, *B. papyrifera*, *F. americana*, and *P. serotina*. These seven choice bioassays were conducted between 15 May and 17 June 2006 using *P. canadensis* individuals from the Vermont early flight population (n = 57) for which only females that laid more than 20 eggs were used (n = 36). Using these data we performed two tests of the variance in response between individuals for each of the hosts.

First, if individuals with greater specificity lay a greater number of eggs on a preferred host and individuals with lower specificity lay eggs more evenly across hosts we should expect that greater variance should be present between individuals for the proportion of eggs laid on the preferred host(s) than for less preferred hosts. We examined the variance between individuals by performing bootstrapped variance estimates for the proportion of eggs laid on each host (1000 non-parametric bootstrap replications) to identify if a higher variance was present for specific hosts. Second, we examined the relationship between hosts by testing for significant correlations between the hosts in the seven choice arenas. A strong preference for a particular host should be evidenced by significant negative correlations between the preferred host and the other common hosts. In contrast, there should be weak or positive correlations amongst the other potential hosts.

Bootstrapped variance estimates, Spearman correlations, and all other analyses presented in this study were performed using the R statistical package V 2.4 (R Development Core Team, 2006).

Differences in oviposition preference hierarchy between *Papilio glaucus* and *Papilio canadensis*

Seven choice assays (2006). In order to compare differences in host rank hierarchy between species and populations, we performed seven choice bioassays identical to the ones mentioned above using females from the *P. canadensis* northern Michigan population (n = 54 set up, 20 used), and the *P. glaucus* Pennsylvania (n = 44 set up, 20 used) and Georgia (n = 27 set up, 15 used) populations. We then combined the proportion

of eggs laid by each butterfly on each host leaf in these seven choice bioassays to those of the *P. canadensis* "early flight" population used above. As responses are likely to be correlated, we performed a Principal Component Analysis (PCA) on this data set to form PCA scores with which we could compare populations along the major axes of variance. Based on the broken stick approach presented in Peres Neto *et al.* (2003), we created a reference to identify which variables (hosts) were relatively more strongly correlated with particular axes. We did this by squaring loadings [the sum of squared loadings across a row (a single variable) is 1] and identifying which loadings exceeded the expected values under the broken stick distribution.

The first three eigenvectors accounted for more than 80% of the variance (Table 3.1), and were therefore used for further analysis. We tested for differences between the four populations along these three main axes by performing Kruskal-Wallis tests on the individual scores for each butterfly along these axes, and contrasted the populations using Bonferroni corrected Mann-Whitney U-tests. We were also interested in testing whether differences for minor hosts were present between the populations, and these were difficult to interpret from the eigenvectors produced. We performed a Varimax rotation on the PCA factor loadings to form axes that were easier to interpret. Using the broken stick model approach mentioned earlier we identified four axes which were not primarily correlated with the proportion of eggs laid on either of the most acceptable hosts, *P. tremuloides* or *L. tulipifera* (axes 5, 6, 7, and 8). We analyzed the individual scores from these four axes as above.

Two choice assays (2006). We were interested in the basis of the difference in responses between *P. canadensis* and *P. glaucus* previously seen in three choice oviposition arenas (Scriber *et al.*, 1991; Figure 3.1). In particular we were interested in whether *P. canadensis* has a lower preference than *P. glaucus* for *L. tulipifera* , and if *P. canadensis* has a greater specificity for *P. tremuloides* than *L. tulipifera* . To test for these differences we conducted two-choice tests consisting of combinations of *L. tulipifera* (Lt), *P. serotina* (Ps), and *P. tremuloides* (Pt) between 21 June 2006 and 22 July 2006 on females from the early flight (EF) and Pennsylvania (PA) populations. Only individuals that laid more than 10 eggs were used for the analysis. In total, 21 EF females and 25 PA individuals were placed in arenas with TT and BC (15 and 18 used, respectively). The EF and PA individuals that successfully laid eggs were placed into arenas with QA and BC; nine EF and four PA individuals laid more than 10 eggs. An additional eight EF and 15 PA individuals were placed in arenas with QA and BC, of which six EF and six PA laid more than 10 eggs. The 14 EF and 10 PA individuals were analyzed together. Finally eight EF and 12 PA individuals were placed in arenas with QA and TT, of which four EF and 11 PA laid more than 10 eggs. To test for differences we contrasted the proportion of eggs laid on each host separately for our two choice bioassays using Bonferroni corrected Mann-Whitney tests.

Table 3.1. Factor loadings for the first three principal component axes of the proportion of eggs laid in seven choice bioassays for the *Papilio canadensis* early flight" (n = 36) and northern Michigan (n = 20) populations, and the *Papilio glaucus* Pennsylvania (n = 20) and Georgia populations (n = 15).

	Principal component axis		
Leaf	1[2]	2	3
L. tulipifera	0.680[1]	0.622[1]	0.03
L. benzoin	-0.054	-0.152	-0.289
P. tremuloides	-0.699[1]	0.503[1]	0.296
P. trifoliata	0.022	-0.307	-0.333
B. papyrifera	-0.016	-0.058	-0.135
F. americana	0.184	-0.473[1]	-0.768[1]
P. serotina	-0.105	-0.125	-0.330
Other	-0.012	-0.011	-0.006
% Variance Explained	51.7 %	18.9%	11.0%

[1]Values higher than expected under the broken-stick model.
[2]Scores between populations for that axis were significantly different between populations (Kruskal-Wallis $P<0.05$).

Seven choice assays (1993-1998). Here we include data from seven choice bioassays performed in 1993 and 1996 using a slightly different host array to compare oviposition responses of F1 hybrids. The methodology used in these bioassays was similar to that used in 2006, with the exception of a small difference in the host array used. In these bioassays all hosts remained the same except that instead of *B. papyrifera*, the invasive species buckthorn, *Rhamnus cathartica* L. (Rhamnaceae) was used in 1993 and 1996. In 1993, 13 females collected in Alaska placed in the oviposition arrays laid more than 20 eggs, and in 1996, three females from Kentucky, 35 females from Ohio, and 30 hybrid females laid more than 20 eggs. In 1998, two females from Charlevoix Co., one female from Dickinson Co., and four females from Emmett Co., laid more than 20 eggs. Due to the small sample sizes and high variance among individuals from the *P. canadensis* populations we limit this data to a qualitative comparison.

Ovposition behavior of the "late flight" population.

The oviposition behavior of the "late flight hybrid population" was contrasted to populations of *P. canadensis* and *P. glaucus* using three choice oviposition bioassays consisting of *L. tulipifera*, *P. serotina*, and *P. tremuloides* conducted during the months of May, June, and July of 2003, 2004, and 2005. A total of 124 *P. canadensis* females from the early flight population, 35 individuals from the late flight hybrid population, 34 individuals from the *P. glaucus* Georgia population, 40 individuals from the *P. canadensis* northern Michigan population, and 118 individuals from the *P. glaucus* Pennsylvania population were set up. Data were expressed as proportions of eggs laid on each host and only those that laid more than 20 eggs were used (94, 29, 25, 27, and 93

females for the "early flight", "late flight", Georgia, northern Michigan, and Pennsylvania populations, respectively). As with the seven choice assays, we were interested in comparing populations along the major axes of variance, so we performed Kruskal-Wallis tests on the scores from the first three axes of a PCA performed on the combined proportion data of the five populations (which accounted for >99% of the variance), and contrasted the populations using Bonferroni corrected Mann-Whitney U-tests. As with seven choice assays we used the broken stick model approach to identify which axes were more strongly associated with particular variables.

Results

Papilio canadensis oviposition preference

Variance estimates were higher for *P. tremuloides* than for any other host (Table 3.2) supporting the notion that there is a preference for *P. tremuloides*. Spearman correlations between hosts in seven choice arenas for *P. canadensis* indicated no correlations between host pairs that did not include *P. tremuloides*, *L. tulipifera,* or *F. americana*. Significant negative correlations and weak correlations were found between the number of eggs laid on *P. tremuloides* and the other hosts, as was predicted if a preference for *P. tremuloides* was present, again supporting that there is a preference for *P. tremuloides*. An unexpected response was the significant positive correlation between the number of eggs laid on *L. tulipifera* and *F. americana.* This correlation is likely a reflection of a reduced specificity for *P. tremuloides* being exhibited as an increased proportion of eggs laid on the other preferred hosts in the oviposition hierarchy.

Table 3.2. Bootstrapped variance estimates of the proportion of eggs laid by the *Papilio canadensis* "early flight" population and the Spearman correlations between the proportion of eggs laid for the three hosts that indicated any significant correlations performed of the same population.

		Spearman correlations		
Plant	Variance ± SE	*P. tremuloides*	*L. tulipifera*	*F. americana*
Populus tremuloides	0.057 ± 0.017	1	x	x
Liriodendron tulipifera	0.026 ± 0.007	-0.75[1]	1	x
Fraxinus americana	0.027 ± 0.014	-0.72[1]	0.54[1]	1
Ptelea trifoliata	0.006 ± 0.001	-0.18	0.29	0.09
Betula papyrifera	0.008 ± 0.002	-0.01	0.16	-0.07
Lindera benzoin	0.009 ± 0.003	-0.06	-0.04	-0.07
Prunus serotina	0.014 ± 0.008	0.24	-0.33	-0.16
Other	0.001 ± 0.0003	0.31	-0.35	-0.11

[1]Significant correlations ($P<0.05$).

Differences in oviposition preference hierarchy between *Papilio glaucus* and *Papilio canadensis*

Seven choice assays (2006). The first three axes of the PCA performed on the seven choice oviposition data of the "early flight", northern Michigan, Pennsylvania, and Georgia populations accounted for 85% of the variance in the data. The first principal component was primarily correlated to the number of eggs laid on *L. tulipifera* and *P. tremuloides* (Table 3.1), with signs in opposite directions. Individual scores along this axis were significantly different among populations (χ^2= 29.79, d.f. = 3, P < 0.001), and Mann-Whitney U-tests indicated significant differences between the *P. canadensis* populations and the *P. glaucus* populations (Table 3.3). The second axis was primarily correlated in one direction with *L. tulipifera* and *P. tremuloides* and in the opposite direction with *P. trifoliata* and *F. americana* . This second axis appears to relate to differences in preference for secondary hosts relative to the preferred hosts of the two species. No significant differences were observed between populations along this axis (χ^2 = 3.44, d.f. = 3, P = 0.33). Finally, the third axis was primarily correlated with *F. americana* in one direction and a weaker correlation with *P. trifoliata* in the other direction and as with the previous axis no difference was observed among populations (χ^2 = 0.23, d.f. = 3, P = 0.97).

Table 3.3. Differences between the *Papilio canadensis* (*Pc*) early flight" (n = 26) and northern Michigan (n = 20) populations, and the *Papilio glaucus* (*Pg*) Pennsylvania (n = 20) and Georgia populations (n = 15) for the individual scores from the first axis of the PCA and the eighth axis of the Varimax rotation performed on the proportion of eggs laid on seven choice arenas.

Population	Principal component 1	Varimax axis 8
Early flight (*Pc*)	A	A
Northern MI (*Pc*)	AB	AB
Pennsylvania (*Pg*)	C	B
Georgia (*Pg*)	BC	B

Populations with the same letters within a column indicate no significant differences from Bonferroni corrected Mann-Whitney tests ($P<0.05$)

Varimax rotation yielded five axes (axes 4, 5, 6, 7, and 8, Table 3.4) that were not strongly correlated to either *L. tulipifera* or *P. tremuloides*. Axis 4 was primarily correlated to *P. trifoliata*, Axis 7 was primarily correlated to *P. serotina*, and axis 6 was primarily correlated to *B. papyrifera* and *F. americana*. There were no significant differences between the populations for these three axes ($\chi^2 = 3.72$, d.f. = 3, P = 0.29; $\chi^2 = 5.88$, d.f. = 3, P = 0.12; and $\chi^2 = 4.23$, d.f. = 3, P = 0.24, respectively). Axis 5 was primarily correlated to *P. trifoliata* and *B. papyrifera*, and although a Kruskal-Wallis test identified a significant difference between populations ($\chi^2 = 10.83$, d.f. = 3, P = 0.013), Mann-Whitney tests failed to detect significant differences between populations. In contrast, significant differences between populations ($\chi^2 = 13.49$, d.f. = 3, P = 0.004; Table 3.4) were observed along axis 8 which was primarily correlated with *B. papyrifera* and *F. americana* in opposite directions.The proportion of eggs laid on each host by each population is represented in Figure 3.2.

Two choice assays (2006). Results from two-choice oviposition assays indicated significant differences between the *P. canadensis* "early flight" and *P. glaucus* Pennsylvania populations. The proportion of eggs laid on *L. tulipifera* was significantly different between the "early flight" and Pennsylvania populations and within each population between the *L. tulipifera -P. tremuloides* and *L. tulipifera -P. serotina* oviposition arrays (Figure 3.3A). Similarly, we found significant differences between the two populations in the proportion of eggs laid on *P. serotina* , but we only found

Table 3.4. Axes formed by Varimax rotation of the factor loadings for the principal component axes of the proportion of eggs laid on seven-choice bioassays for the *Papilio canadensis* early flight (EF) (n = 36) and northern Michigan (MI) (n = 20) populations, and the *Papilio glaucus* Pennsylvania (PA) (n = 20) and Georgia (GA) populations (n = 15)

	Varimax axis							
Leaf	1[3]	2[3]	3[3]	4	5	6	7	8[3]
Liriodendron tulipifera	0.70[1]	-0.68[1]	-0.18	0.02	0.11	-0.02	-0.01	0.05
Lindera benzoin	0.50[2]	0.62[1]	-0.47[2]	0.31	-0.13	0.06	0.01	-0.15
Populus tremuloides	0.30	0.03	0.77[1]	0.33	-0.33	0.14	0.01	-0.29
Ptelea trifoliata	-0.15	-0.04	0.06	0.79[1]	0.48[2]	-0.12	-0.01	0.31
Betula papyrifera	0.03	0.07	0.07	-0.15	0.68[1]	0.52[2]	-0.01	-0.49[2]
Fraxinus americana	0.08	0.08	0.06	-0.06	-0.12	0.74[1]	0.06	0.65[1]
Prunus serotina	0.13	0.11	0.13	-0.13	0.16	-0.17	0.93[1]	0.10
Other	-0.35	-0.35	-0.35	0.35	-0.35	0.35	0.35	-0.35
% Variance Explained	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%

[1]Values above the average largest value under the broken-stick model.
[2]Values above the average second largest value under the broken-stick model.
[3]Scores between populations for that axis were significantly different between populations (Kruskal-Wallis $P<0.05$).

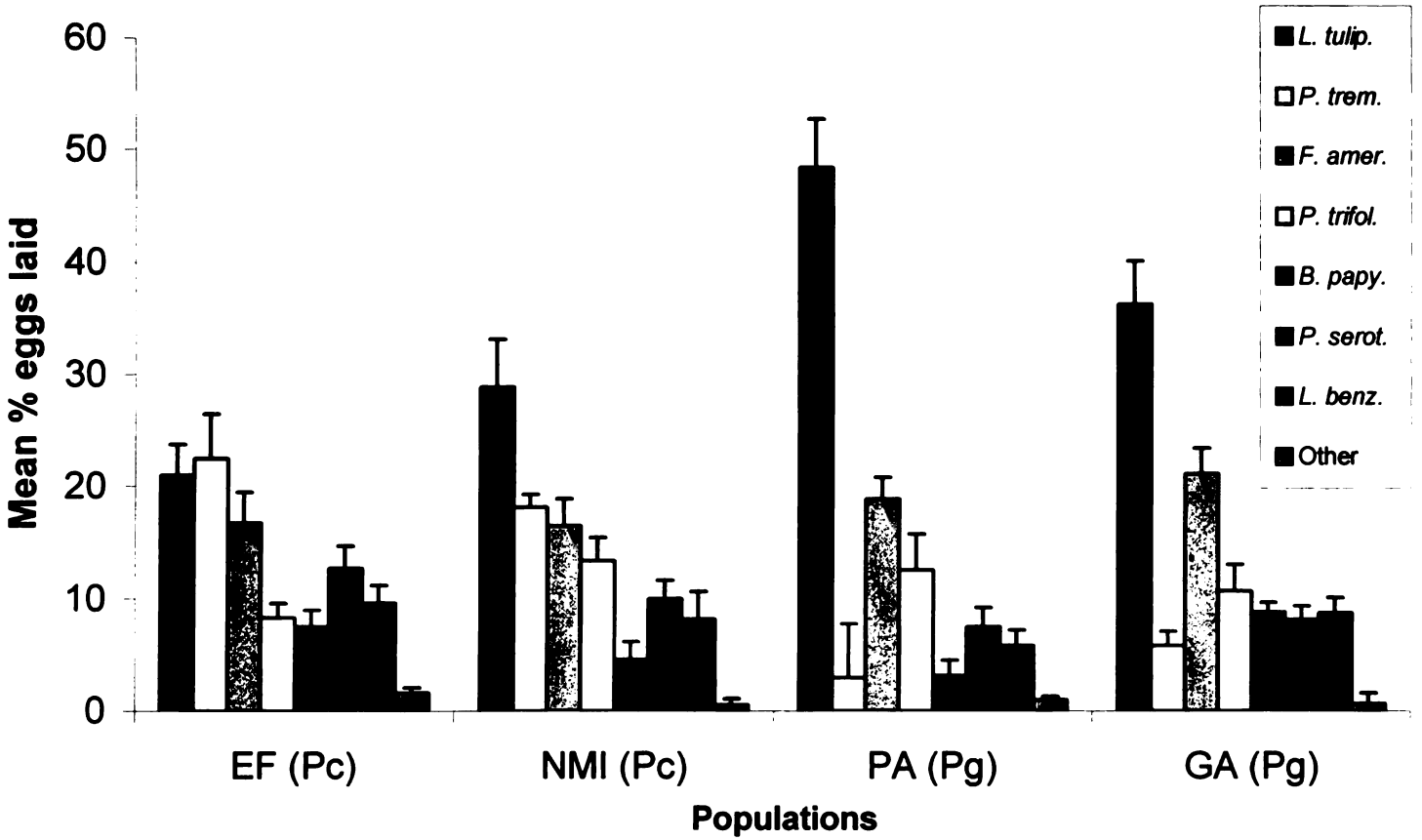


Figure 3.2. Mean percentage + SE of eggs laid by the 36 *Papilio canadensis* (*Pc*) females from the "early flight"(EF) population, 20 *P. canadensis* females from the northern Michigan (NMI) population, 20 *Papilio glaucus* (*Pg*) females from the Pennsylvania population, and 15 *P. glaucus* females from the Georgia (GA) population. Leaves present in the bioassay were *L. tulipifera*, *P. tremuloides*, *F. americana*, *P. trifoliata*, *B. papyrifera*, *P. serotina*, and *L. benzoin*. The "other" category represents eggs laid on the paper or plastic of the oviposition arena.

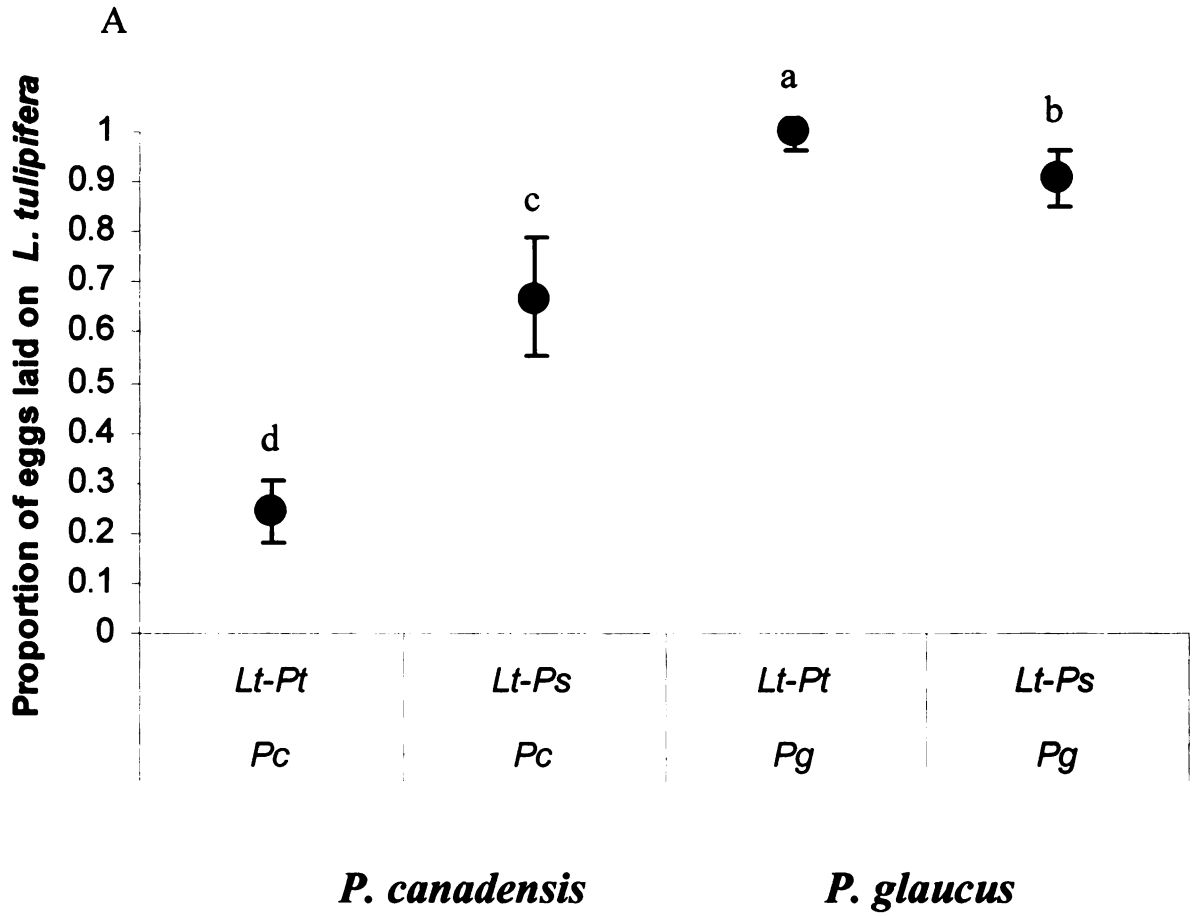


Figure 3.3. (A) Median proportion of eggs laid, with interquartile ranges, by *Papilio glaucus* (*Pg*) and *Papilio canadensis* (*Pc*) butterflies on *L. tulipifera* (*Lt*) in two-choice assays. Leaves present in the bioassays were *L. tulipifera* and *P. serotina* (*Ps*) or *L. tulipifera* and *P. tremuloides* (*Pt*). For each leaf the pairwise differences between the proportions of eggs laid on it in the different arrays (host and butterfly set-ups) were analyzed using Mann-Whitney tests. Arrays with the same letter did not have a significant difference at a Bonferroni corrected $\alpha<0.05$.

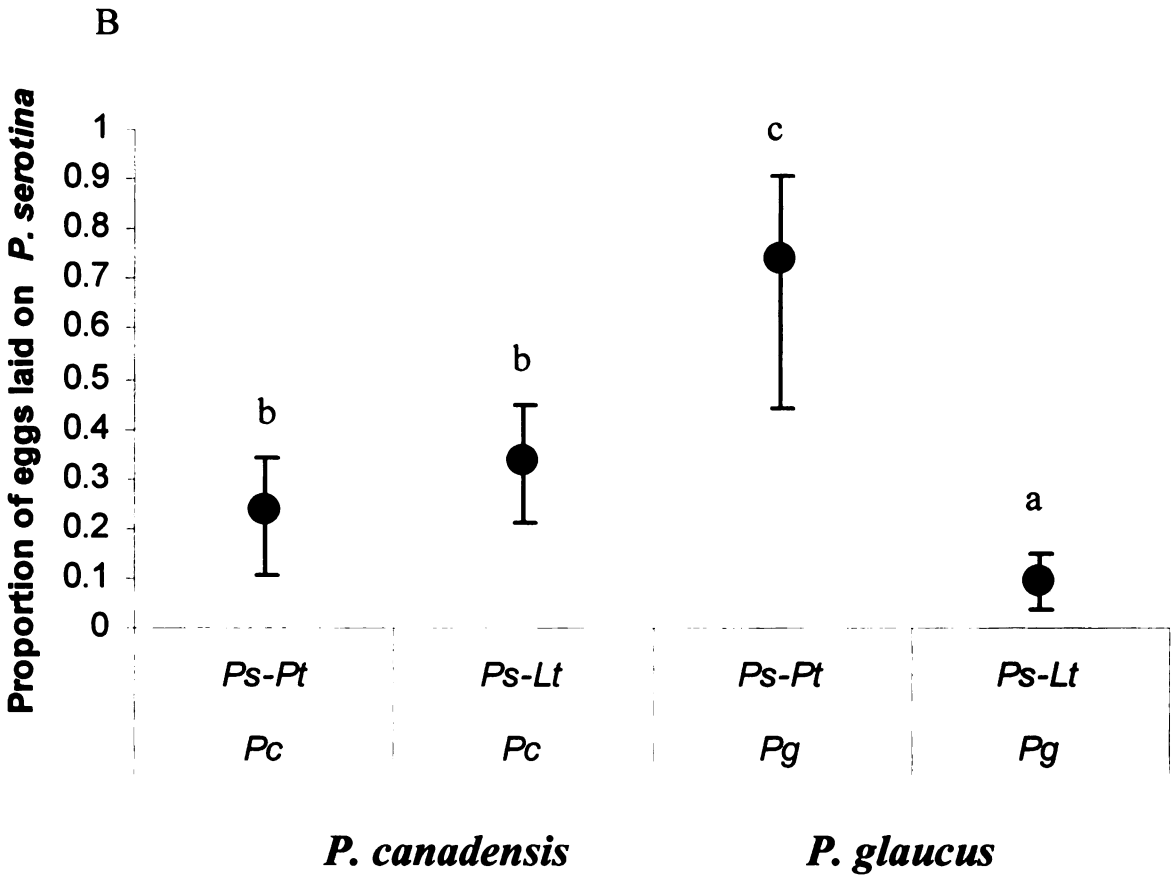


Figure 3.3. (B) Median proportion of eggs laid, with interquartile ranges, by *P. glaucus* and *P. canadensis* butterflies on *P. serotina* in two-choice assays. Leaves present in the bioassays were *P. serotina* and *L. tulipifera* or *P. serotina* and *P. tremuloides*. For each leaf the pairwise differences between the proportions of eggs laid on it in the different arrays (host and butterfly set-ups) were analyzed using Mann-Whitney tests. Arrays with the same letter did not have a significant difference at a Bonferroni corrected $\alpha<0.05$

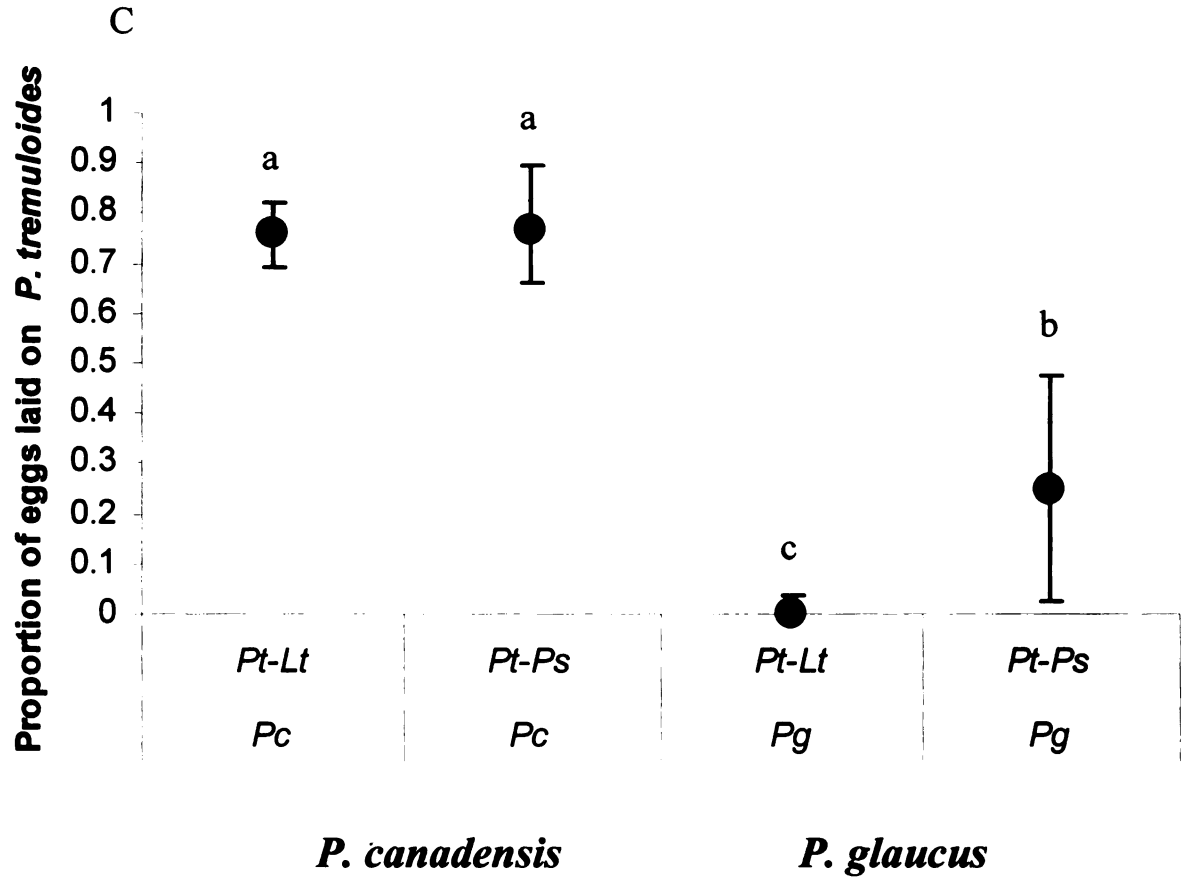


Figure 3.3. (C) Median proportion of eggs laid, with interquartile ranges, by *P. glaucus* and *P. canadensis* butterflies on *P. tremuloides* in two-choice assays. Leaves present in the bioassays were *P. tremuloides* and *L. tulipifera* or *P. tremuloides* and *P. serotina*. For each leaf the pairwise differences between the proportions of eggs laid on it in the different arrays (host and butterfly set-ups) were analyzed using Mann-Whitney tests. Arrays with the same letter did not have a significant difference at a Bonferroni corrected $\alpha<0.05$.

differences between arrays for the Pennsylvania population (Figure 3.3B). Finally, for the proportion of eggs laid on *P. tremuloides*, we again saw significantly different responses between populations, but only significant differences between arrays for the Pennsylvania population (Figure 3.3C).

Seven choice assays (1993-1998). Oviposition behavior of the females from 1993, 1996, and 1998 indicated similar results to those found in three choice assays (Scriber *et al.*, 1991; Scriber, 1994). Here we again observed that hybrids had a similar oviposition pattern to that of the fathers genotype. In this case F1 hybrids of *P. canadensis* females by *P. glaucus* males had a similar oviposition pattern to that of *P. glaucus* (Figure 3.4).

Ovpositional behavior of the "late flight" population.

The first three axes of the PCA performed on the combined three choice oviposition data of the "early flight", northern Michigan, Pennsylvania, Georgia, and "late flight" populations accounted for >99% of the variance in the data. The first principal component was primarily correlated to the proportion of eggs laid on *L. tulipifera* and a weaker correlation with the proportion of eggs laid on *P. tremuloides* (Table 3.5), with signs in opposite directions. This axis is likely a representation of the lower acceptance of *L. tulipifera* characteristic of the difference between *P. glaucus* and *P. canadensis*. Individual scores along this axis were significantly different between populations (χ^2 = 32.02, d.f. = 4, P<0.001), and Mann-Whitney U-tests indicated significant differences between the "early flight" and northern Michigan *P. canadensis* populations and the two *P. glaucus* and "late flight" populations (Table 3.6).

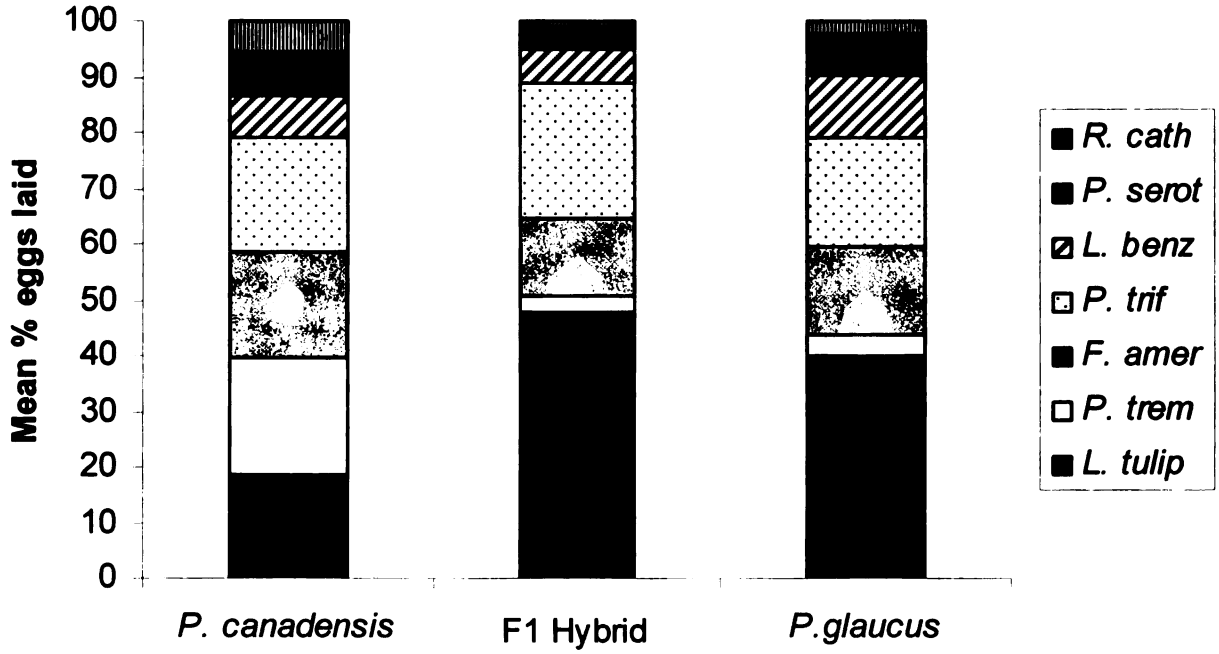


Figure 3.4. Mean percentage of eggs laid by *Papilio glaucus* (n = 38) from Ohio and Kentucky, *Papilio canadensis* (n = 20) from Alaska and northern Michigan, and F1 hybrids (n = 30) from *P. canadensis* mothers and *P. glaucus* fathers. Leaves present in the bioassay were *L. tulipifera*, *P. tremuloides*, *F. americana*, *P. trifoliata*, *L. benzoin*, *P. serotina*, and *R. cathartica*.

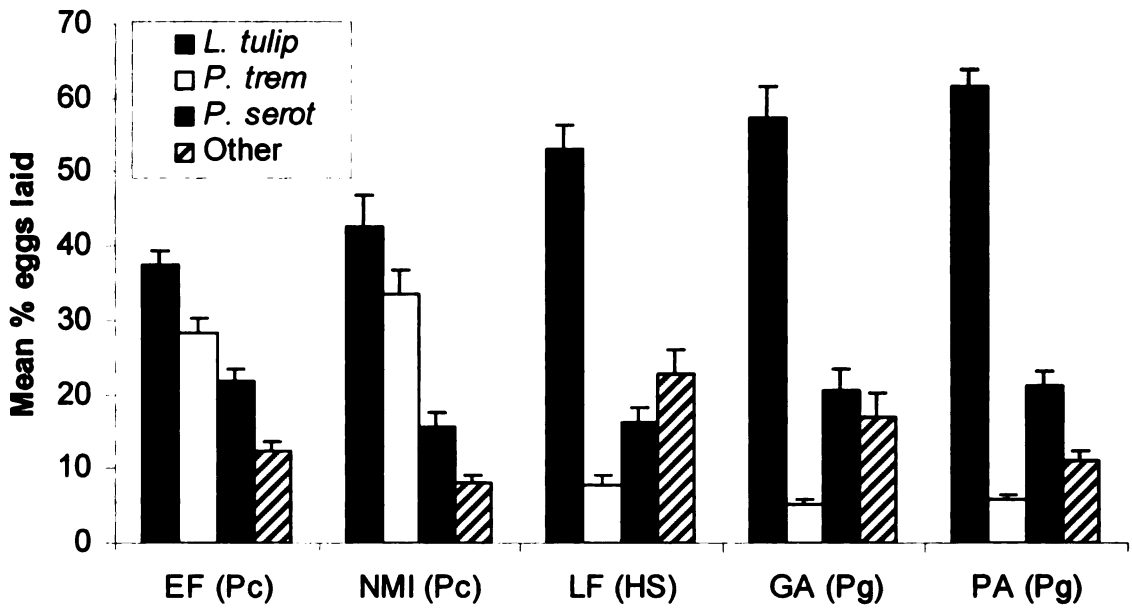


Figure 3.5. Mean percentage + SE of eggs laid by 94 *Papilio canadensis* (Pc) females from the "early flight" population (EF), 27 *P. canadensis* females from the northern Michigan population (NMI), 29 females from the "late flight" (LF) hybrid swarm (HS) population, 25 *Papilio glaucus* (Pg) females from the Georgia population (GA), and 93 *P. glaucus* females from the Pennsylvania (PA) population. Leaves present in the bioassay were *L. tulipifera*, *P. serotina*, and *P. tremuloides*. The "other" category represents eggs laid on the paper or plastic of the oviposition arena.

Table 3.5. Factor loadings for the first three principal component axes of the proportion of eggs laid on three choice oviposition assays for the *Papilio canadensis* early flight" (n = 94) and northern Michigan (n = 27) populations, the *Papilio glaucus* Pennsylvania (n = 93) and Georgia populations (n = 20), and the "late flight" hybrid swarm population (n = 29).

	Principal component axis		
	1[3]	2[3]	3[3]
Leaf			
L. tulipifera	0.844[1]	-0.149	0.124
P. serotina	-0.255	0.653[2]	0.509[2]
P. tremuloides	-0.451	-0.713[1]	0.197
Other	-0.138	0.209	-0.829[1]
% Variance Explained	61.1%	21.0%	17.9%

[1]Values above the average largest value under the broken-stick model.
[2]Values above the average second largest value under the broken-stick model.
[3]Axes for which individual scores were significantly different between populations.

Table 3.6. Differences between the *Papilio canadensis* (*Pc*) early flight" (n = 94) and northern Michigan (n = 27) populations, the *Papilio glaucus* (*Pg*) Pennsylvania (n = 93) and Georgia populations (n = 20), and the "late flight" hybrid swarm population (HS) (n = 29) for the individual scores from the first two axis of the PCA performed on the proportion of eggs laid on three choice arenas. Populations with different letters within a column indicate significant differences from Bonferroni corrected Mann-Whitney tests ($P<0.05$)

	Axis	
Population	1	2
Early flight VT (*Pc*)	B	B
Northern MI (*Pc*)	B	C
Pennsylvania (*Pg*)	A	A
Georgia (*Pg*)	A	A
Late flight VT (HS)	A	A

The second axis was primarily correlated in one direction with *P. tremuloides* and to a lesser extent *P. serotina* in the opposite direction (Table 3.5). Significant differences were observed between populations along this axis ($\chi^2_4 = 55.45$, $P < 0.001$), not surprisingly as the acceptance of *P. tremuloides* is also a characteristic difference between *P. canadensis* and *P. glaucus*. However, significant differences along this axis were not only observed between the "early flight" and northern Michigan and the other populations, but also between the "early flight" and northern Michigan population (Table 3.6). The differences observed between the "early flight" and northern Michigan populations and the other three populations are likely to be a representation of the very low number of eggs laid on *P. tremuloides* by individuals other than *P. canadensis*. On the other hand the difference between the "early flight" and the northern Michigan population are likely due to differences in specificity between the two populations.

The third axis is difficult to interpret as it is primarily correlated with the proportion of eggs laid on non-leaf portions of the arena and to a lesser extent on *P. serotina* in the opposite direction (Table 3.5). This axis could be construed as a representation of the types of "mistakes" made by insects with a certain level of residual excitation, as this axis represents the proportion of eggs laid on a host low on the hierarchy (Figure 3.2) and eggs laid on non-leaf portions of the arena. As seen with the previous axes, significant differences were observed among populations ($\chi^2 = 30.67$, d.f. = 4, $P<0.001$; Table 3.6).

Discussion

The selection pressure for specialization as a function of encountering the wrong host has been noted since the first model used to explain the predominance of specialist insects was developed (Levins & MacArthur, 1969). The probability that *P. canadensis* females will encounter most of the toxic hosts used by its sister species (e.g., *L. tulipifera*) are very low. It is therefore feasible that due to the low likelihood of encountering acceptable toxic hosts, a lower overall specificity may be sufficient to incorporate a host for which there previously was very limited acceptability (e.g., *P. tremuloides*). Under such a scenario, the oviposition differences between *P. glaucus* and *P. canadensis* seen in three choice oviposition assays (Scriber *et al.*, 1991; Figure 3.1) could simply be a change in specificity. Courtney *et al.*(1989) noted that changes in specificity are far more likely to occur due to the higher level of genetic variance likely to exist for specificity while host rank hierarchies are more likely to remain stable. In *P. glaucus* this appears to be the case, as genetic variance between populations is seen for specificity of oviposition preference (Bossart & Scriber, 1995), while its host plant preference hierarchy is maintained throughout its range (Mercader & Scriber, 2005). However, in this study we detected a shift in the preference rank hierarchy between *P. canadensis* and *P. glaucus* (Tables 3.2, 3.4 and Figure 3.2). This illustrates an example of a shift in the oviposition preference rank hierarchy during the polyphagous stage of a lineage, without reducing the acceptance rates of secondary hosts.

Whether this shift in preference rank hierarchy between *P. glaucus* and *P. canadensis* was due to an acceptance of plants in the Salicaceae by *P. canadensis* or a

loss of acceptance for plants in the Salicaceae by *P. glaucus* is difficult to determine. Within the *Papilio glaucus* group, another generalist species closely related to *P. glaucus* and *P. canadensis, Papilio rutulus,* also naturally uses plants in the Salicaceae (Scriber, 1996), and it is not possible to resolve whether the acceptance of the Salicaceae by ovipositing females arose independently in *P. rutulus* and *P. canadensis* or once in the ancestor of *P. rutulus, P. canadensis,* and *P. glaucus* and was then subsequently lost in *P. glaucus.* However, ovipositing females of both *P. multicaudatus* [closely related and basal to *P. glaucus, P. canadensis,* and *P. rutulus* (Zhakharov *et al.*, 2004)] and *P. rutulus* in oviposition arenas containing suitable host plants will accept *L. tulipifera* (RJ Mercader and JM Scriber unpubl.), although it is toxic to their larvae (Scriber, 1996). In contrast, *P. multicaudatus,* like *P. glaucus,* has a very low acceptance rate for *P. tremuloides*, suggesting that within the *P. glaucus* group acceptance of Magnoliaceae hosts by ovipositing females is likely primitive, while acceptance of Salicaceae hosts is likely derived.

In addition, the oviposition behavior observed for *P. glaucus* and *P. canadensis* was consistent with what would be expected for the acceptance of a new host as described by Courtney *et al.*(1989). In particular, we observed the acceptance of a new host and the ancestral host by *P. canadensis,* and very limited acceptance of the novel host by *P. glaucus* (Figure 3.2). Surprisingly, the acceptance of *P. tremuloides* did not significantly alter the response of *P. canadensis* for most of the secondary hosts also used by *P. glaucus* (Figure 3.2 and Tables 3.2, 3.3, and 3.4). While we found significant differences in the seven choice assays between populations for axes relating to *L. tulipifera* and/or *P. tremuloides* (Tables 3.2, 3.3, and 3.4), the only difference along any

axes we found, that was not primarily related to *P. tremuloides* or *L. tulipifera*, was the difference between the "early flight" *P. canadensis* population and the two *P. glaucus* populations along the eighth Varimax axis (Tables 3.3 and 3.4). This axis was primarily related to *F. americana* in one direction and to a lesser extent *B. papyrifera* in the opposite direction, which may be an indication of a higher acceptance of *F. americana* in *P. glaucus* and higher *B. papyrifera* acceptance in *P. canadensis*. Unfortunately, we are unable to distinguish if this small exception is due to the strong correlation between *L. tulipifera* and *F. americana* or if it is an independent response. However, even with this small exception our results strongly indicate that the primary difference between the two species is a reduced acceptance of *L. tulipifera* and a preference for *P. tremuloides* in *P. canadensis* relative to *P. glaucus*. Our two-choice oviposition assays also supported this notion. In these assays *P. canadensis* females laid a significantly lower number of eggs on *L. tulipifera* and a significantly higher proportion of eggs on *P. tremuloides* than *P. glaucus* (Figure 3.3). It is interesting to note that no significant differences were found amongst *P. canadensis* females in the number of eggs laid on *P. serotina* in assays containing *L. tulipifera* or *P. tremuloides* (Figure 3.3B).

The *Papilio glaucus* group is unique amongst the Papilionidae in its high degree of polyphagy and by its "escape" from the host specializations on tropical plant families, such as Lauraceae, Magnoliaceae, and Rutaceae, which along with the Aristolochiaceae, Annonaceaee, and Apiaceae constitute the diet of 90-95% of all Papilionidae (Scriber, 1996). The shift in host plant use in the *Papilio glaucus* group (e.g., to include the Salicaceae, Betulaceae, Rosaceae, and Oleaceae) may have been facilitated by the alleles coding for these preference differences having likely been Z-linked (as seen for the

Salicaceae in *P. canadensis* (Scriber, 1994; Figure 3.4)). Z-linked differences in oviposition preference rank hierarchies may be an important component of butterfly host associations as has been observed in other related and unrelated groups (Thompson, 1988, 1993, 1994; Janz, 1998; Nygren *et al.*, 2006). As proposed for female preference of male ornaments (Reeves & Pfennig, 2003), Z-linked traits under positive selection expressed only by females in species with a ZZ/ZO sex determination (female heterogamety) are less likely to be lost to genetic drift, and therefore it is more likely for these rare alleles to become fixed in populations.

Scriber (1994), using F1 *P. glaucus* and *P. canadensis* reciprocal hybrids, found the difference in three choice oviposition trials (similar to the ones reported here) were Z-linked. Similarly, in this study we also found corresponding responses in seven choice assays between *P. glaucus* and hybrids with *P. canadensis* mothers and *P. glaucus* fathers (Figure 3.4). This result further supports the notion that the primary difference observed between *P. glaucus* and *P. canadensis* is their relative preferences for *L. tulipifera* and *P. tremuloides*, which appears to be Z-linked. It is unknown whether the Z-linked oviposition difference is due to a single Z-linked preference for *P. tremuloides* in *P. canadensis* and a separate preference for *L. tulipifera* in both species of uncertain inheritance, or two Z-linked traits; one for the acceptance of *P. tremuloides* and *L. tulipifera* in *P. canadensis* and one for only the acceptance of *L. tulipifera* in *P. glaucus*. Previous studies using three choice oviposition assays of *P. glaucus,* similar to the ones reported here, suggest that the acceptance of *P. tremuloides* may be independent from specificity for *L. tulipifera* (Mercader & Scriber, 2005). In particular, they indicate a very weak negative relationship between the proportion of eggs laid on *L. tulipifera* (a

measure of specificity) and *P. tremuloides* (adjusted $r^2 = 0.09$) compared to the negative relationship between the proportion of eggs laid on *L tulipifera* and *P. serotina* (adjusted $r^2 = 0.60$).

In addition to differences between *P. glaucus* and *P. canadensis,* differences in oviposition profiles have been noted between populations of *P. canadensis* in natural field conditions (Scriber, 2002a) and choice assays not containing *L. tulipifera* (Scriber & Lederhouse, 1992; Scriber, 1996b). The differences in oviposition profiles constituted reductions in the proportion of eggs laid on *P. tremuloides* and increases in the proportion of eggs laid on *F. americana* in populations located in areas where local cold temperatures, "Cold Pockets", significantly reduced the quality of the available *P. tremuloides*. These observed shifts in oviposition profiles appear to represent multiple independent changes in host rank hierarchy in these "Cold Pocket" populations. However, the significant correlation between the proportions of eggs laid on *L. tulipifera* and *F. americana* and the significant negative correlation between the proportion eggs laid on *P. tremuloides* and these two hosts in *P. canadensis* (Table 3.2), hints at an alternative mechanism by which this shift may have occurred. A reduced specificity for *P. tremuloides* would likely be reflected as a preference for *L. tulipifera* or *F. americana* in *P. canadensis*, which in oviposition assays without *L. tulipifera* would be represented as an apparent preference for *F. americana.* In this case it is likely that changes in specificity for *P. tremuloides* have led to an apparent shift in host rank hierarchy for the locally highest quality host. Whether changes in specificity and host ranking are simply differential expressions of the same genes, as has been proposed for *Polygonia c-album* (Janz, 2003), or due to different genes is unknown.

Irrespective of the exact mechanism behind the differences in specificity and host preference ranking, the combination of the acceptance of a novel host family (Salicaceae) and the potential for changes in specificity provide a likely avenue for diversification of *P. canadensis*. One potential reason for the lack of divergence observed is the low selection pressure for plant specialization predicted for populations of univoltine species with relatively long growing seasons (Scriber & Lederhouse, 1992) and the absence of *L. tulipifera* in the vast majority of *P. canadensis* range. In addition to not encountering ancestral hosts, it is likely that even if acceptance of toxic hosts via contact chemoreception exists, changes in other senses may be likely to change the functional response in the field. For example, responses to extracts from dried leaves of *L. tulipifera* elicit three-fold higher electroantennagram responses in *P. glaucus* than *P. canadensis* (Mercader *et al.* In Press). It is possible that changes in the perception of visual or olfactory cues could lead to functionally specialized populations in the field which we are unable to distinguish in our oviposition arenas.

The recently described "late flight" hybrid swarm population (Scriber & Ording, 2005; Scriber *et al.*, 2007) provides another avenue that may lead to specialization in this group. This population is thought to have formed due to recent increased introgression of *P. glaucus* alleles into *P. canadensis* populations leading to Z-chromosome recombinant hybrids which resulted in an allochronically separated population. This population represents a mixture of the *P. canadensis* and *P. glaucus* genomes including a recombined Z-chromosome with Z-linked allozymes diagnostic of both *P. glaucus* and *P. canadensis* (Scriber & Ording, 2005). *A priori*, we would have expected either a *P. canadensis* oviposition pattern or a mixture of *P. canadensis* and *P. glaucus* oviposition

patterns amongst females. This expectation was due to another Z-linked *P. canadensis* trait, obligate diapause, being essential for survival in this region where two generations are not possible. In this "late flight" population we found the oviposition profile in three choice assays to be that of *P. glaucus.* In particular we found significant differences with *P. canadensis* and not *P. glaucus* populations along PCA axis 1 and 2 (Table 3.6). Axis 1 was primarily related to proportion of eggs laid on *L. tulipifera*, while axis 2 was primarily related to the proportion of eggs laid on *P. tremuloides* in one direction and to a lesser extent the number of eggs laid on *P. serotina* in the opposite direction (Table 3.5). Together these axes represent the main difference between *P. glaucus* and *P. canadensis* observed in the seven choice assays (a lowered preference for *L. tulipifera* and a preference for *P. tremuloides*) and indicate that the "late flight" hybrid swarm population has a *P. glaucus* oviposition profile.

This oviposition profile has significant implications for the ecology and potential diversification of this population as plants in the Magnoliaceae are absent in or north of the historical hybrid zone where the "late flight" population occurs (Scriber & Ording, 2005; Scriber *et al.*, 2007), making *L. tulipifera* an unlikely host. However, *F. americana* is abundant where the "late flight" occurs, and although we did not assay this population for *F. americana*, both *P. glaucus* and *P. canadensis* use *F. americana* as a host. Therefore it is highly likely that the "late flight" hybrid swarm population may be primarily utilizing *F. americana*, which if it becomes/remains reproductively isolated could lead to specialization on *F. americana.* Future studies on the host use of this *Papilio* hybrid swarm population and populations from the putative species *P. appalachiensis* (Pavulaan & Wright, 2002) may help elucidate whether such a change is

likely and provide an avenue to study the process of specialization and possible constraints to specialization in these organisms.

CHAPTER 4

HYBRIDIZATION LEADS TO A HOST SHIFT IN A POLYPHAGOUS BUTTERFLY SIBLING SPECIES PAIR.

Abstract

Recent increased movement of *Papilio glaucus* into historically *P. canadensis* territory, due to climate warming, has led to the formation of an allochronically separated hybrid population with a delayed emerging phenotype or "late flight" first observed in 1999 in the Battenkill River Valley, at the New York-Vermont border, USA. Here we assess how the recombination of the parental genomes leading to the formation of this phenotype may have facilitated another major ecological shift, host use divergence. We first contrast the ovipositional profiles of the "late flight" population to that of the parental species *P. glaucus* and *P. canadensis*. Second we contrast larval survival and growth on five hosts of the "late flight", a *P. canadensis* population, a *P. glaucus* population, and another population from the Northern edge of the hybrid zone. Our results indicate that the ovipositional preference of this hybrid swarm is identical to that of the introgressing parental species, *P. glaucus*. Due to the absence of the preferred hosts of *P. glaucus* (*Liriodendron tulipifera* L. and *Ptelea trifoliata* L.) where the "late flight" occurs, this ovipositional pattern implies an apparent functional shift onto a secondary host of both parental species, *Fraxinus americana* L. In contrast, the larval host use abilities represent a mixture of *P. glaucus* and *P. canadensis*, indicating divergence in larval host use abilities has not taken place. However, high genetic variability (CV_G) is present for

growth on *F. americana* in the "late flight" hybrid swarm and tradeoffs for larval performance on the preferred hosts of the parental species are evident (*L. tulipifera* and *Populus tremuloides* Michx.); indicating a strong potential for specialization in larval host use abilities to occur. This current scenario represents an instance where a shift in a major ecological trait, host use, is likely occurring as a byproduct of a shift in an unrelated trait (delayed emergence) leading to partial reproductive isolation.

Introduction

Recent anthropogenic changes to the environment are creating novel selection pressures upon organisms that have already lead to several examples of rapid evolution (e.g. examples reviewed in Thompson 1998; Palumbi 2001). These changes not only affect individual populations or species, but can affect entire communities by altering the interactions between organisms. This has been seen in the interactions between plant feeding insects and their hosts due to plant invasions (Strauss *et al.* 2006; Carroll 2007; Singer *et al.*2008) and land use change (Singer *et al.*2008). The interactions between plants and plant feeding insects are vital for community composition and function (e.g. Weisser and Siemann 2004), and are arguably the most common interaction between multicellular organisms in terrestrial systems (plants and insects compose approximately 40% of terrestrial organisms; Price 2002). Due to the ubiquity of plant insect interactions and their important role in community composition, understanding the potential (and documented) impacts of environmental change on plant-insect associations is of significant importance.

The high diversity of plant feeding insects is believed to be due to their radiation onto the wide chemical diversity of plants (e.g. Ehrlich and Raven 1964; Mitter *et al.*1988; Futuyma 1989; Weingartner *et al.*2006). In particular, this divergence is believed to be the result of the colonization of novel hosts, making novel niches available for adaptive radiation to take place (e.g. Jermy 1984; Janz *et al.*; 2001; Jermy and Szentsi 2003; Percy *et al.*2004; Braby and Trueman 2006; Lopez- Vaamonde *et al.* 2006; Wheat *et al.*2007). Although specialization would appear to be an evolutionary dead end, transitions between generalist and specialist forms within lineages are believed to be common (Janz *et al.*2001; Nosil 2002; Scriber *et al.* 2008b) and a hypothesis of oscillating host expansion and specialization has been proposed to help explain the great diversity of plant feeding insects (Janz *et al.*2006; Janz and Nylin 2008). However, how the genetic variation leading to these host shifts arises and is maintained is not clearly understood for most systems.

One probable source of genetic variation leading to isolation and host shifts is hybridization, as has been recently observed in *Rhagoletis* fruit flies (Schwarz *et al.* 2005). The notion that hybridization can form novel phenotypes leading to speciation is not a new idea, and can be traced as far back as Linnaeus (Arnold 1997; Coyne and Orr 2004). However, while botanists have acknowledged the importance of hybridization as an evolutionary force generating novel genotypes, zoologists have historically viewed hybridization primarily as maladaptive (reviewed in Arnold 1997; Coyne and Orr 2004). In recent years the role of hybridization as an evolutionary force generating novel genotypes has been gaining support, and begun to be accepted in animal taxa as well (e.g. reviews by Seehausen 2004; Mallet 2007). The importance of hybridization as an

evolutionary force centers on the view that the formation of novel, transgressive, phenotypes by hybridizing organisms allows for the colonization of open niches, facilitating speciation and adaptive radiations (Seehausen 2004; Mallet 2007).

Implicit in hybridization is the recombination of genomes, which will undoubtedly lead to the recombination of most traits in organisms, not only those that may potentially lead to reproductive isolation. If hybridization leads to a reproductively isolated or partially isolated population, the evolutionary trajectory of such a population would be constrained by the genetic variability inherited from the parental populations. This may or may not be an equal representation of both parental populations. The importance of phylogenetic constraints on insect radiations has been widely recognized in the study of plant-insect interactions (e.g. Ehrlich and Raven 1964; Price 2008). In the case of reproductively isolated (or partially isolated) populations resulting from hybridization events, the recombination of traits that takes place may lead to novel constraints and/or the release from constraints (relative to the parental populations) unrelated to the trait(s), leading to isolation.

A recent instance of a hybridization event leading to a transgressive phenotype has occurred in the Battenkill River Valley, at the New York-Vermont border, USA. In this region, recent climate warming has greatly increased the gene movement across a hybrid zone delineating the range limits of the Eastern Tiger Swallowtail, *Papilio glaucus,* and the Canadian Tiger Swallowtail, *Papilio canadensis* (Scriber 2002). Prior to recent warming trends, this region was near the Southern edge of the univoltine *P. canadensis* range, where only one generation of *P. canadensis* or *P. glaucus* could develop starting at the beginning of the growing season. However, after 1998 this region

warmed significantly and became capable of supporting an early emerging population (typical *P. canadensis* or *P. glaucus* phenotype) as well as a potentially late emerging population (Scriber *et al.*2008a). The increased movement in *P. glaucus* traits into the region has produced a transgressive phenotype with delayed emergence, or "late flight", forming an allochronically separated hybrid swarm population that can exploit this novel thermal zone (Scriber and Ording 2005; Scriber *et al.*2008a).

The host use abilities of this "late flight" are relatively unknown as the two parental species *P. glaucus* and *P. canadensis* are both highly polyphagous species, but have differing ovipositional preferences (Scriber *et al.*1991; Mercader and Scriber 2007) and larval host use abilities (Scriber 1996). Preliminary evidence from three choice oviposition assays indicate that the "late flight" population has a similar ovipositional profile to *P. glaucus* (Mercader and Scriber 2007), and the ability to survive on hosts of both *P. glaucus* and *P. canadensis* (Scriber *et al.* 2008a). In this study we assess how the formation of this delayed emerging phenotype may have affected host use traits in this "late flight" population. We use bioassays for both oviposition and larval host use abilities on plants of variable quality for both parental species. We first contrast the ovipositional profiles of the "late flight" population to *P. glaucus* and *P. canadensis* using oviposition assays containing host plants of *P. glaucus* and/or *P. canadensis* from seven different plant families. Secondly we contrast survival and growth of the "late flight", *P. glaucus, P. canadensis,* and a population located at the Northern edge of the *P. glaucus* and *P. canadensis* hybrid zone on leaves from five plant families. Finally, we present a quantitative genetic analysis for larval host use abilities of the "late flight"

population using estimates of the broad sense heritability and coefficients of genetic variation from larval growth assays on five hosts.

Materials and Methods

Insect and Plant Sources

Female butterflies from the "late flight" population used in seven choice oviposition assays were a combination of wild-caught females and individuals reared from eggs laid by wild-caught females. All collections of "late flight" individuals took place in mid to late July 2006 (mothers of mass reared individuals) and 2007 (wild-caught females) in the Battenkill River Valley area at the NY/VT border, USA (43° N latitude and 73° W longitude) as described in Scriber & Ording (2005). All individuals were mass reared on *P. serotina* in sleeves placed on tree branches in the Battenkill River Valley in 2006. Reared females were mated by hand pairing them to a single wild caught male or lab reared male (not from the same sib group), wild females were assumed to have been mated in the wild and were subsequently not hand paired. Eggs laid by females ovipositing in our seven choice bioassays were collected daily and the emerging larvae were used in our growth assays as described below.

Larvae from the remaining populations were collected from adult females ovipositing in three-choice or two-choice arenas. In addition, the ovipositional patterns in seven choice assays are well established for the *P. canadensis* and *P. glaucus* populations used (Mercader and Scriber 2005, 2007, 2008b). Larvae of *P. glaucus* from a Georgia population were obtained from the eggs of wild captured females collected in Oglethorpe Co., GA, USA in 2007 (34° N latitude and 83° W longitude). Larvae of *P. canadensis* from a Northern Michigan population were obtained from eggs of field collected females in 2007 from Cheboygan and Emmet Counties MI, USA, (45° N

latitude and 84° W longitude). Finally, larvae from a population in the Michigan hybrid zone were obtained from field collected females in 2007 from Isabella County MI, USA, (43° N latitude and 84° W longitude) located at the Northern edge of the historical hybrid zone (Scriber *et al.*2003). All wild caught butterflies were transported in coolers to our MSU laboratory where they were fed a 15-20% honey water solution prior to placement in the oviposition arenas.

Leaves were collected from trees growing in Ingham County, MI, USA, from locations known to be pesticide free. These were placed in aquapicks inside plastic bags, and kept in a refrigerator for no more than 3 days to ensure leaf quality and turgor remained high. Leaves used in the assays were collected from at least four different trees, with the exception of *L. benzoin* and *B. papyrifera* for which two and one plants were used, respectively, since plants known to be pesticide free were more limited for these two hosts.

Oviposition Assays

We placed butterflies in oviposition arenas containing leaves from plants in seven different families of varying larval suitability for *P. canadensis* and *P. glaucus* (see above); *L. tulipifera* (Magnoliaceae), *P. tremuloides* (Salicaceae), *F. americana* (Oleaceae), *P. trifoliata* (Rutaceae), *P. serotina* (Rosaceae), *B. papyrifera* (Betulaceae), and *L. benzoin* (Lauraceae). These seven choice bioassays were conducted between 15 May and 24 July 2007 using individuals from the Vermont "late flight" population (n = 50). Only females that laid more than 20 eggs were utilized for later analysis (n = 26). The general methods used were similar to those described by Scriber (1993); arenas consisted of clear round plastic containers placed on a rotating platform (approximately 10 revolutions per h) in front of incandescent lights on a L4:D4 hrs photocycle to maximize egg laying. Leaves of each plant species were placed in floral aquapicks to

maintain turgidity. Butterflies were fed a honey-water solution daily and allowed to oviposit until they were too weak to fly (4-12 days). The number of eggs laid on leaves of each plant or non-leaf portions were counted daily and the leaves replaced as necessary. No leaf remained for more than 2 days in the assays.

Ovipositional responses from these individuals were contrasted to those of *P. glaucus* individuals from Lancaster Co. in South-Eastern PA, USA (40° N latitude and 76° W longitude) and *P. canadensis* individuals from the "early flight" in the Battenkill River Valley area at the New York/Vermont border in 2006 for a previous study using identical methodology (Mercader and Scriber 2007). Unfortunately we were unable to procure sufficient specimens from these populations in 2007 to run the assays simultaneously.

Larval Growth Assays

Eggs collected from ovipositing females were maintained at 25 °C and checked at least twice daily for hatching larvae. To increase within family replication we did not include all hosts used in the oviposition arenas, and limited our larval assays to the five most commonly used hosts of *P. glaucus* and *P. canadensis; L. tulipifera* (Magnoliaceae), *P. tremuloides* (Salicaceae), *F. americana* (Oleaceae), *P. trifoliata* (Rutaceae), and *P. serotina* (Roseaceae). We allocated all recently hatched neonate larvae onto the five host plants using a fine camel hair brush. Leaf petioles were supported in rubber-capped water-filled vials to maintain turgor and leaves were checked daily to ensure high quality. All larvae were maintained at a constant 25 °C with a 18:6 hrs (Light:Dark) photoperiod. After three days larvae were checked for survival and instar achieved. After six days all

larvae were checked again for survival and instar achieved. They were then frozen at -20 °C. At the conclusion of the experiment all larvae were weighed to the nearest 0.1 mg while still frozen to prevent water loss.

In total, 1388 larvae from 36 families of the "late flight" population, 401 larvae from 13 families of the Northern Michigan population, 251 larvae from 8 families of the Georgia population, and 417 larvae from 11 families of the Michigan hybrid zone population were set up. For our growth studies we only used families for which at least five larvae survived after six days. This reduced the number of larvae used in the growth assays to 945 larvae from 25 families of the "late flight" population, 310 larvae from 11 families of the Northern Michigan population, 172 larvae from 7 families of the Georgia population, and 326 larvae from 10 families of the Michigan hybrid zone population.

Statistical Analyses

Population contrasts

Oviposition: We contrasted the overall ovipositional profiles of *P. glaucus, P. canadensis* "early flight", and the "late flight" hybrid swarm by performing a one-way non-parametric MANOVA (NP MANOVA) on the proportion of eggs laid on each leaf by individual butterflies in the seven choice bioassays. MANOVA has been shown to be an effective tool to differentiate oviposition preference ranking using proportion data from choice trials similar to our own (Thompson, 1988; Bossart and Scriber, 1995). However, we were unable to satisfy assumptions of MANOVA through transformations and therefore used a Non Parametric MANOVA (or Permutation MANOVA) as described by Anderson (2001), and implemented in the PAST program (Hammer *et al.*, 2001), using

the Bray-Curtis distance matrix and 10,000 iterations. We then used pair-wise NP MANOVAs as post-hoc tests to determine significant differences between the three populations. Significance for pairwise NP MANOVAs was accepted at Bonferroni corrected $P<0.05$.

Survival: Survival between populations was analyzed separately for each host as repeated measures analyses using Generalized Linear Mixed Models (GLMM) (Family = Binomial, logit link) in the lme4 package (Bates and Sarkar 2007) of R (R Development Core Team 2007). In these analyses the state of the larvae after six days (alive or dead) was the dependent variable, population the fixed effect, and family was considered the random effect. By declaring family as the random effect, family was considered our "subject", thereby constraining our analysis to comparisons of family performances, rather than individual performances, between populations. We performed pairwise contrasts of populations in these analyses using Tukey's HSD for pairwise contrasts in the multcomp package (Hothorn *et al.*2007).

Growth: Here we were interested in contrasting the relative performance of individuals from families from different populations on each host, rather than overall size differences between families or populations. In order to contrast the relative performance of individuals on different hosts we took the Z-score of each individual weight by subtracting the family average weight (on all hosts) from each individual weight (on a single host) and dividing this value by the standard deviation for each family (on all hosts). We then contrasted the Z-scores between populations for each host as for survival

using GLMM (Family = Gaussian) with population as the fixed effect and family as the random effect and Tukey's HSD for pairwise contrasts.

Analyses Within the Late Flight

We were interested in two primary comparisons within the "late flight" hybrid swarm population.

1) Is there high genetic variation for growth on the different hosts?

As this "late flight" population is a known hybrid swarm from two species of organisms with known differences in host use we are inclined to expect significant variation in host use abilities. However, this may not necessarily be the case for all (or any) of the hosts assayed. While significant differences have been observed in growth rate on *P. glaucus* and *P. canadensis, P. canadensis* has not shown significant differences in growth rate between populations in Michigan and Alaska (Ayres 1991), indicating a potentially limited variation in detoxification abilities in *P. canadensis.* Secondly, it is unknown if the recombination events and strong divergent selection on the X(Z)-chromosome (leading to the formation of the "late flight") population selectively removed variation leading to reduced variation in host use ability for particular hosts. Finally, we were unaware if there would be lower or greater variation for growth on *L. tulipifera* and *P. tremuloides* relative to the common hosts *F. americana* and *P. serotina,* or for *P. trifoliata.*

Quantitative genetic analyses of hybrids can be difficult to interpret, particularly when considering the F_1 generation. Gordon (1999) derived the quantitative genetic expectations for F_1 hybrid swarm populations and identified that additive genetic

variance and narrow sense heritability cannot be defined for these populations. However, he found the genotypic variance and broad sense heritability to be defined for these populations and useful determinants of genetic advance from selection. In addition, Gordon (2000) derived the theoretical expectations for an allogamous F_2 population, derived from F_1 crosses and determined that allogamous F_2 populations behaved in an identical fashion to the randomly fertilized populations on which classical quantitative genetic analysis is based. In this study we do not estimate additive genetic variation, as we are using full-sib variance estimates, and are deriving our estimates for a likely F_{2+} population. Therefore, our main obstacle is the same as that for any other study based of a full sib design; we are unable to separate additive and non-additive genetic components.

In order to determine the level of genetic variation within the "late flight" we performed Mixed Model analyses for each host again using the lme4 package and Markov Chain Monte Carlo estimates of variability for the parameters measured using the mcsamp function in the arm package (Gelman *et al.*2008) for R. In these analyses we included date of measurement as a fixed effect (to account for potential differences in leaf quality as the season progressed) and family as a random effect. Using these data we measured broad sense heritability H^2, the genetic coefficient of variation CV_G, and residual coefficient of variation CV_R following Houle (1992). As we were only able to sample full sibling families our estimates differ from those of Houle (1992), in our use of total genetic variance (V_G) rather than purely additive genetic variance (V_A). The use of V_G rather than V_A will likely lead to an inflated value relative to the additive genetic variance coefficient CV_A and a deflated value for the CV_R.

2) Are there significant interactions between host use abilities?

In order to determine if there were significant interactions between host use abilities we performed two tests. First we ran linear Mixed Models using the lme4 package (Bates and Sarkar 2007) for each host pair combination using larval weight as the dependent variable, family as the random factor, host as the fixed factor, and date as a covariate. We ran these models with and without an interaction between family and host and performed a log likelihood ratio test for each host pair combination between the model with an interaction between family and host and the model without the interaction. Our second test consisted of taking the average family Z-score (calculated as above) for each host and performing Spearman correlations between family averages on each host pair combination.

Results

Oviposition Assays

The ovipositional responses of *P. glaucus* were virtually identical to those of the "late flight" hybrid swarm population, whereas those of the *P. canadensis* "early flight" population were different (Figure 4.1). Not surprisingly the NP MANOVA performed found a significant overall difference in the ovipositional behavior of these three populations ($F_{2,69} = 11.8$, $P<0.001$), and pairwise comparisons indicated significant differences in the overall ovipositional responses between the "early flight" population and the *P. glaucus* and "late flight" populations (Table 4.1).

Larval Growth Assays

Survival: The results for 6-day survival for the four populations sampled are summarized in Figure 4.2. Log likelihood ratio tests between models with family as a random effect and with or without population as a fixed effect indicated a significant effect of population for all hosts tested (*L. tulipifera* $\chi^2 = 28.6$, $P < 0.001$; *P. tremuloides* $\chi^2 = 35.8$, $P < 0.001$; *F. americana* $\chi^2 = 18.0$, $P < 0.001$; *P. serotina* $\chi^2 = 20.2$, $P < 0.001$; *P. trifoliata* $\chi^2 = 10.1$, $P = 0.02$). Within *L. tulipifera* we found significant differences in survival between all populations except between the "late flight" and the Michigan Hybrid zone population (Figure 4.2). Of particular note is the significant difference observed between the "late flight" population and the Georgia *P. glaucus* population, indicating reduced survival on *L. tulipifera* in this hybrid swarm relative to the pure *P. glaucus* type (Figure 4.2). A similar result was observed for *P. tremuloides* with the exception that no significant difference was observed between the Michigan hybrid zone population and the Northern Michigan population (Figure 4.2). As with *L. tulipifera* the "late flight" population had a lower survival on *P. tremuloides* than the reference *P. canadensis* population (Figure 4.2). However, for *F. americana, P. serotina,* and *P. trifoliata* significant differences were only found between the Northern Michigan population and the "late flight" population (Figure 4.2). This may reflect a lower overall survival in the "late flight" population. However, lower sample sizes for the Georgia and Hybrid zone populations preclude us from being able to discern whether this difference is due to high survival in the Northern Michigan population or low survival in the "late flight" population.

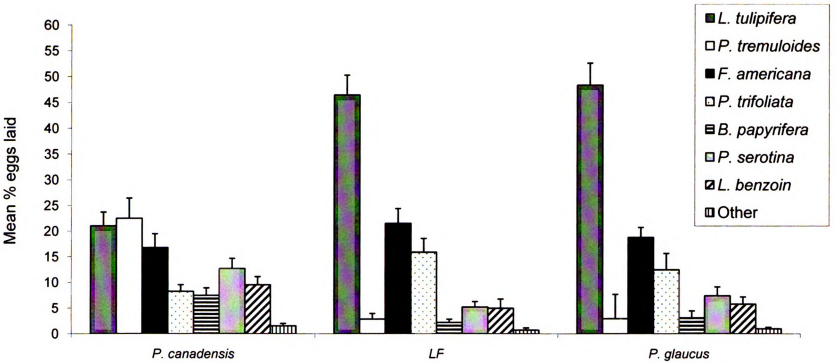


Figure 4.1. Mean percentage + SE of eggs laid by the 36 *Papilio canadensis* females from the "early flight" population (2006), 26 females from the "late flight" hybrid swarm (2007), and 20 *Papilio glaucus* (*Pg*) females from Pennsylvania (2006). Leaves present in the bioassays were *L. tulipifera*, *P. tremuloides*, *F. americana*, *P. trifoliata*, *B. papyrifera*, *P. serotina,* and *L. benzoin*. The "other" category represents eggs laid on the paper or plastic of the oviposition arena.

Table 4.1. P-values for pairwise Non-Parametric MANOVA's contrasting the ovipositional behavior in seven choice arenas. Upper triangle represent uncorrected P-values and the lower triangle Bonferroni corrected P-values.

	Population		
Population	Late Flight	*P. canadensis*	*P. glaucus*
Late Flight		<0.001	0.518
P. canadensis	**<0.001**		<0.001
P. glaucus	**1**	**<0.001**	

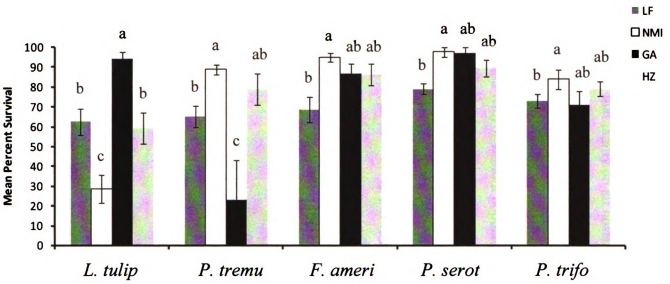


Figure 4.2. Mean percent larval survival ± SE of the "late flight" population (LF), of the *P. canadensis* Northern Michigan population (NMI), of the *P. glaucus* Georgia population, and of the Michigan hybrid zone population (HZ). The five leaf species on which assays were performed are *L.tulipifera, P. tremuloides, F. americana, P. serotina,* and *P. trifoliata.* Within each host species population means followed by the same letter are not significantly different at the $p<0.05$ level (Tukeys HSD).

Larval growth (Between Populations): A summary of the relative growth of the caterpillars on each host as measured by family Z-Scores is illustrated in Figure 4.3. These Z-Scores represent the growth of larvae in each family on one host relative to the overall performance of that family on all hosts; therefore they are quantitative measures of host suitability ranking within families. They are by necessity also only representative of those caterpillars that survived. Log likelihood ratio tests between models with family as a random effect and with or without population as a fixed effect indicated a significant effect of population for *L. tulipifera* ($\chi^2 = 14.7$, $P = 0.002$), *P. tremuloides* ($\chi^2 = 21.4$, $P < 0.001$), *F. americana* ($\chi^2 = 11.7$, $P = 0.009$), and *P. trifoliata* ($\chi^2 = 10.4$, $P = 0.015$), but not for *P. serotina* ($\chi^2 = 2.7$, $P < 0.44$). For *L. tulipifera* significant differences were observed between the Northern Michigan population and the other three populations, but no significant differences between any of the other populations (Figure 4.3). For *P. tremuloides* significant differences were observed between most populations with the exception of the Michigan hybrid zone population compared to the "late flight" or the Northern Michigan populations (Figure 4.3). Finally, significant differences within *P. trifoliata* were only observed between the Georgia *P. glaucus* population and the other three populations (Figure 4.3).

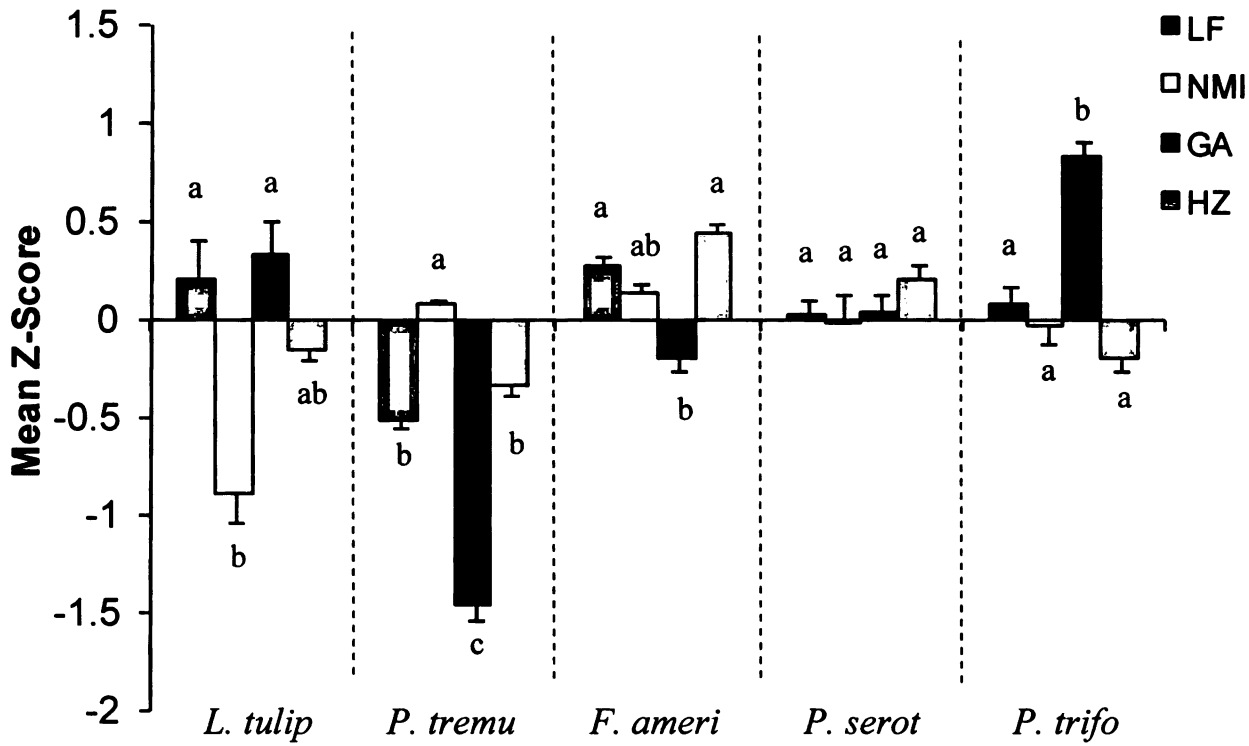


Figure 4.3. Mean Z-score for larval growth + SE of the "late flight" population (LF), of the *P. canadensis* Northern Michigan population (NMI), of the *P. glaucus* Georgia population, and of the Michigan hybrid zone population (HZ). The five plant species on which assays were performed are *L.tulipifera, P. tremuloides, F. americana, P. serotina,* and *P. trifoliata.* Within each host species population means followed by the same letter are not significantly different at the $p<0.05$ level (Tukeys HSD).

Estimates of genetic variance for larval performance: We found fairly low estimates of broad sense heritability for larval growth on all hosts measured (Table 4.2). However, we found relatively high coefficients of genetic variability (CV_G) for growth on *P. tremuloides* (17.6) and *P. trifoliata* (23.4), lower for *F. americana* (11.1) and *P. serotina* (11.01), and lowest for *L. tulipifera* (3.81). Although the CV_G for growth on *L. tulipifera* was lower than that for the other four hosts it is still similar to those measured for adult body size in *Drosophila* (Wayne *et al.*1997). Morphological traits have often been found to have high heritability estimates and low coefficients of genetic variance (Houle 1992). In contrast, life history traits tend to have low heritability estimates and high coefficients of genetic variability (Houle 1992). As in other studies (Houle 1992) the low heritability and high CV_G in this study is likely due to the presence of high residual variance observed for growth on all hosts measured (CV_R; see Table 4.2). These results indicate that in this hybrid swarm population there is a high level of genetic variability for this important life history trait (larval host use ability), but not equal levels of genetic variability for the ability to use different hosts.

In addition to high levels of genetic variability, we detected significant family by host interactions indicating significant genotype by environment correlations. In particular, log likelihood ratio tests between models with family as a random effect, host as a fixed effect, and with or without host by family interactions indicated a significant interaction effect for *L. tulipifera* and *P. tremuloides* ($\chi^2 = 6.18$, P = 0.046), *L. tulipifera* and *P. trifoliata* ($\chi^2 = 10.6$, P =0.005), *P. tremuloides* and *P. trifoliata* ($\chi^2 = 18.8$, P <0.001), and *F. americana* and *P. trifoliata* ($\chi^2 = 11.3$, P =0.004). In contrast, we found

Table 4.2. Summary of variance estimates derived from the "late flight" population for weights (mg) after six days of growth on five plants using GLMMs. Parameters represented are the square root of genetic variance ($\sqrt{V_G}$) and residual variance ($\sqrt{V_R}$) ± standard deviation (from MCMC estimates), and the corresponding coefficient of genetic variance (CV_G), coefficient of residual variance (CV_R), and broad sense heritability (H^2).

	L. tulipifera	***P. tremuloides***	***F. americana***	***P. serotina***	***P. trifoliata***
Families (n)	23 (171)	24 (214)	24 (212)	17 (161)	16 (153)
Mean ± SEM	23.6 ± 1.1	19.3 ± 0.8	26.1 ± 0.9	22.7 ± 0.9	22.2 ± 1.0
$\sqrt{V_G}$ ± Stdev	0.9±1.1	3.4±0.9	2.9±1.6	2.5±1.8	5.2±1.7
$\sqrt{V_R}$ ± Stdev	12.7±0.7	9.4±0.5	10.3±0.5	9.5±0.6	10.3±0.6
CV_G	3.81	17.61	11.11	11.01	23.42
CV_R	53.81	48.7	39.46	41.85	46.39
H^2	0.05	0.12	0.1	0.12	0.2

no significant interaction effects between *L. tulipifera* and *P. serotina* ($\chi^2 = 0.6$, P =0.74), *L. tulipifera* and *F. americana* ($\chi^2 = 0.29$, P =0.86), *P. tremuloides* and *F. americana* ($\chi^2 = 3.5$, P =0.17), *P. tremuloides* and *P. serotina* ($\chi^2 = 0.92$, P =0.63), *F. americana* and *P. serotina* ($\chi^2 = 0.9$, P =0.64), and *P. serotina* and *P. trifoliata* ($\chi^2 = 4.59$, P =0.1).

Spearman correlations between family means for the Z-scores indicated significant negative correlations between *L. tulipifera* and *P. tremuloides* (Spearman correlation= -0.56, P=0.006) and between *P. tremuloides* and *P. trifoliata* (Spearman correlation= -0.54, P=0.041). No significant correlations were found for any other host pair combination (Figure 4.4). The lack of significant correlations between *L. tulipifera* and *P. trifoliata* and between *F. americana* and *P. trifoliata,* is an indication that the significant interactions found above were random or weakly directional (i.e. some were positive and some were negative). In contrast, the significant negative correlations between *L. tulipifera* and *P. tremuloides* and between *P. tremuloides* and *P. trifoliata* indicate that the genotype X environment interactions observed were strongly directional, an indication of potential tradeoffs in host use ability between *P. tremuloides* and *L. tulipifera* and *P. trifoliata.*

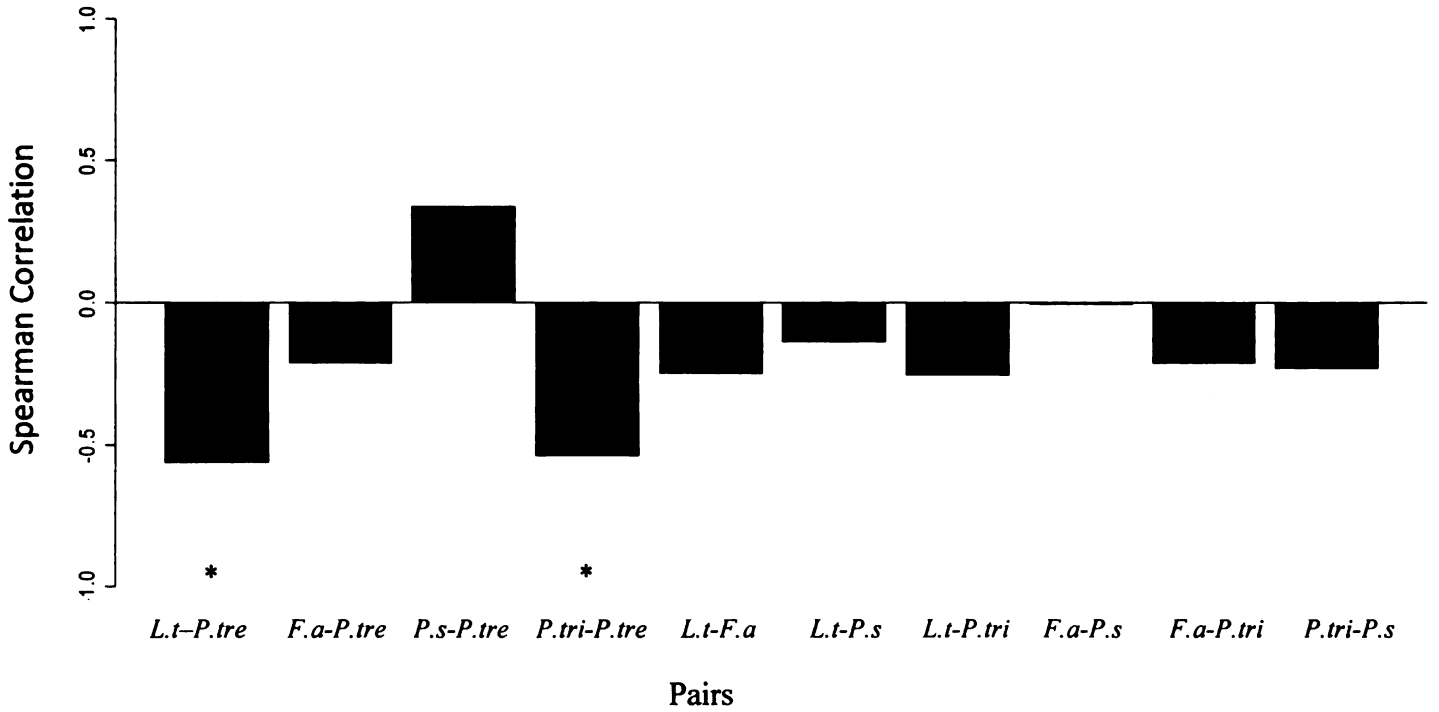


Figure 4.4. Spearman correlations of mean family Z-scores for growth, performed between pairs of five host species. Host labels are *L. tulipifera* (*L.t*), *P. tremuiloides* (*P.tre*), *F. americana* (*F.a*), *P. serotina* (*P.s*), and *P. trifoliata* (*P.tri*)

Discussion

Ovipositional Preference

In plant feeding insects where larvae complete development on a single host, such as most tree feeding insects, the ovipositional behavior of the adult female determines larval host use. For this reason shifts in ovipositional behavior are necessary for host use divergence or specialization to occur in most tree feeding insects. Our results indicate an identical ovipositional profile in the "late flight" hybrid swarm to that of the introgressing parental species, *P. glaucus* (Figure 4.1 and Table 4.1). In particular, the "late flight" does not accept the most common host (*P. tremuloides*) of the parental species (*P. canadensis*) found in this region. In addition, neither the preferred host, *L. tulipifera,* nor the second most common host of *P. glaucus, P. trifoliata,* are present where the "late flight" occurs. In fact, of those hosts on which it laid over 5% of its eggs, only *F. americana* is present in the Battenkill River Valley (United States Geological Survey, http://esp.cr.usgs.gov/data/atlas/little). Therefore a functional shift in host preference is likely taking place in this population towards this secondary host of both parental species. An important consideration is that as global warming proceeds tree distributions will likely be affected. Iverson *et al.* (2008) modeled the potential shift in 134 tree species under six climate scenarios, and indicated the potential for forest types (Oak-Hickory) associated with significant *L. tulipifera* densities to move into the Battenkill River Valley by 2100. However, under all the climate scenarios Iverson *et al.* (2008) modeled *L. tulipifera* is not expected to invade this region, while *F. americana* is expected to increase in abundance (http://www.nrs.fs.fed.us/atlas/tree/tree_atlas.html).

Previous work using three choice assays had also indicated a similar ovipositional behavior of the "late flight" hybrid swarm relative to that of *P. glaucus* (Mercader and Scriber 2007). In the same study, using a combination of seven choice, three choice, and two choice oviposition assays, we determined that the primary difference in the ovipositional behavior of *P. glaucus* and *P. canadensis,* is a Z-linked acceptance of *P. tremuloides* as an ovipositional substrate and a reduced level of specificity in *P. canadensis.* The results presented in the current study along with our previous results indicate that the shift in ovipositional profile in this hybrid swarm, relative to the local parental species, is due to the absence of a Z-linked trait for the acceptance of *P. tremuloides.* This result is striking, because the late-flight also possesses a Z-linked trait present in *P. canadensis* and not *P. glaucus,* obligate diapause, which is essential to prevent a second generation incapable of completing development in the Battenkill River Valley (Scriber and Ording 2005; Scriber *et al.*2008a). It is highly unlikely that this phenotype can be attributed to natural selection, due to the short period of time this population has existed (first observed in 1999 in a regularly sampled region). Therefore the presence of the *P. glaucus* ovipositional behavior and the *P. canadensis* obligate diapause trait on the same chromosome indicate that this shift in ovipositional behavior is likely a byproduct of the recombination event leading to the delayed emergence phenotype that forms the "late flight".

Functional differences in ovipositional behavior due to differing environments, rather than in the underlying behavior, are not unique to this hybrid swarm population. Like *P. canadensis,* the Western species of the *P. glaucus* group, *P. multicaudatus, P. rutulus,* and *P. eurymedon* will also accept *L. tulipifera* as an ovipositional substrate even

though their larvae are incapable of developing on it (Mercader and Scriber 2008b in Press). However, this potential host shift represents the first case for which we know the origin of the genetic variation. Several studies have demonstrated how hybridization can lead to speciation by forming transgressive phenotypes capable of utilizing novel environments (e.g. Riesberg 1997; Schwarz *et al.*2005, 2007; Mavarez *et al.*2006; Gompert *et al.*2007). Here we demonstrate how the recombination leading to a transgressive phenotype, delayed emergence, has also lead to a major ecological shift (host use) unrelated to the novel environment (thermal niche).

Larval survival and growth (between populations)

Both hybrid populations tested had high survival on three host plants tested that were suitable to both parental species, *F. americana, P. serotina,* and *P. trifoliata.* However, we found significantly lower survival for both of these hybrid populations relative to the introgressing parental species, *P. glaucus,* on its preferred host (*L. tulipifera*) relative and significantly lower survival for the "late flight" hybrid swarm relative to the other parental species, *P. canadensis,* on its most common host (*P. tremuloides*) (Figure 4.2). *L. tulipifera* and *P. tremuloides* are also the two hosts for which *P. glaucus* and *P. canadensis* show strong differences in larval survival (Scriber 1996; Figure 4.2). These results demonstrate that the "late flight" population, and to a lesser extent the Michigan hybrid zone population, maintain a mixture of larval host use abilities from both parental species, unlike what is seen for ovipositional behavior.

In addition to differences in survival, we found significant differences in the relative host suitability of the surviving larvae from these populations (Figure 4.3). For

these contrasts we accounted for overall family size differences across hosts by using Z-scores (see Materials and Methods). This approach allowed us to contrast the relative ability to use the five hosts assayed between two species with inherently different growth rates and initial hatching weights and their hybrids. Therefore these results are not contrasts of differences in absolute size between populations, but rather of the relative suitability of each host for the families assayed from each population.

L. tulipifera is known to have a wide variety of defensive compounds particularly sesquiterpene lactones (Doskotch 1983; Lindroth *et al.*1986; Scriber *et al.*1987; Barbosa *et al.*1989), which *P. glaucus* is capable of handling, but *P. canandensis* is not (Lindroth *et al.*1986). The ability of *P. glaucus* to feed on *L. tuilipifera* appears to be at least partly due to its ability to excrete unaltered sesquiterpene lactones (Frankfater *et al.*2005). In this study we found the relative growth of the "late flight" on *L. tulipifera* was positive, indicating it was a comparatively good host. In addition, we found no significant difference in the relative suitability of *L. tulipifera* for *P. glaucus* and the "late flight" population (Figure 4.3), indicating that the surviving "late flight" larvae may have inherited from *P. glaucus* the ability to detoxify or excrete sesquiterpene lactones.

In contrast, for both the "late flight" population and the Michigan hybrid zone population, performance on *P. tremuloides* was negative and significantly lower than for the Northern Michigan *P. canadensis* population (Figure 4.3). The ability to consume *P. tremuloides* by *P. canadensis* appears to be primarily based on the ability to detoxify phenolic glycosides by high esterase activity (Lindroth *et al.*1986; Lindroth *et al.*1988; Scriber *et al.*1989; Scriber *et al.*1999). Studies using backcrosses between *P. glaucus* and *P. canadensis* have indicated that the ability to use *P. tremuloides* acts in a dose

dependent manner, where hybrids have intermediate abilities to detoxify these compounds (Scriber *et al.*1989), as is also seen in these natural hybrid populations.

The likely host for the "late flight", *F. americana,* appeared to be an excellent host for both hybrid populations and *P. canadensis,* but was not as good a host for *P. glaucus* (Figure 4.3). This apparently low performance for *P. glaucus* larvae is likely due to the excellent growth observed on *P. trifoliata,* reducing its Z-score relative to the other three populations. *P. glaucus* is known to have a higher ability to detoxify furanocoumarins present in Rutaceae hosts, such as *P. trifoliata,* than is *P. canadensis* due to cytochrome P-450 enzymes (Li *et al.*2001, 2002, 2003; Mao *et al.*2007). Although no significant difference was observed in the relative suitability of *P. trifoliata* between the "late flight" and *P. canadensis* from Northern Michigan, it is still possible that the *P. glaucus* like cytochrome P-450 enzymes are present in this population, but not in all families (see below).

These results again indicate that the larval host use abilities of the "late flight" population are a mixture of the host use abilities of both parental species. The combination of detoxification abilities suggests that this population currently remains unspecialized in its larval host use abilities. Future specialization will depend on the genetic variability present for these traits (see below)

Estimates of genetic variance for the "late flight" population

The ability to specialize depends on both preference for a host and performance on that host. Ovipositional results indicated that preference in the "late flight" is towards a functional shift onto a secondary host, *F. americana.* Our genetic analysis of larval

weight indicates that while there is a low heritability for growth on *F. americana* there is a relatively high level of genetic variation for this trait (Table 4.2). The low levels of heritability are due to the high levels of residual variance observed, which are an indication that large within-family variation is likely.

In this study we are unable to separate additive and non-additive genetic components due to our use of a full-sib family design. However, the large within-family variation and the intermediate mortality within families on *P. tremuloides* and *L. tulipifera* relative to *P. canadensis* and *P. glaucus* (Figure 4.2) are an indication of non-additive effects segregating out in these hybrid individuals. Within classic quantitative genetics theory non-additive effects are considered to be of little importance. However, recent studies indicate the opposite may be true as non-additive effects have been found to be exceedingly common and of significant importance (Roff and Emerson 2006), including important roles in host race formation and speciation (Carroll *et al.*2001; Wade 2002; Rego *et al. 2007*).

High genetic variation was also found for *P. tremuloides, P. serotina,* and *P. trifoliata,* but relatively low genetic variation for *L. tulipifera* (Table 4.2). The lower genetic variability observed for *L. tulipifera* may be due to a relatively uniform detoxification system in *P. glaucus* (although this is unlikely, given results in Bossart and Scriber 1995), mortality of individuals exhibiting alternate enzymatic combinations, or a limited introgression of detoxification abilities of *P. glaucus.* At this point we are uncertain as to why we should see relatively lower levels of genetic variation for growth on *L. tulipifera* compared to the ability to growth on the other four hosts (Table 4.2).

However, although the level of genetic variability for growth on *L. tulipifera* is lower, it exists and is of a similar degree to that found for morphological characters in *Drosophila* spp. (Wayne *et al.*1997). In fact, we see a significant genotype by host interaction in the ability to use *L. tulipifera* and *P. tremuloides* (Results). Spearman correlations between family Z-score means for these two hosts indicate a strong negative correlation (Figure 4.4). The primary difference in host use that has been observed between *P. glaucus* and *P. canadensis* is a distinct difference in their abilities to detoxify Magnoliaceae (*L. tulipifera*) and Salicaceae (*P. tremuloides*) hosts (Scriber 1996; Scriber *et al.*2003). The presence of this strong negative genetic correlation found here indicates that a tradeoff in host use ability may have maintained the historically sharp boundary for host use ability along the *P. glaucus* and *P. canadensis* hybrid zone. In addition, we also found significant genotype by host interactions between *P. trifoliata* and *L. tulipifera, F. americana,* and *P. tremuloides* (Results). However, there were no significant correlations observed between *P. trifoliata* and *L. tulipifera* or *F. americana* (Figure 4.4), indicating a weak (or lack of) directionality for these interactions. In contrast, there was significantly negative correlation between *P. trifoliata* and *P. tremuloides.* The significant number of genotype by host interactions including *P. trifoliata* is indicative of the presence of *P. glaucus* and *P. canadensis* like cytochrome P450 enzymes, however this needs to be tested.

An important consideration for the likely evolutionary trajectory of the detoxification abilities of the "late flight" hybrid swarm population is the ovipositional profile described above. In particular, this hybrid swarm is unlikely to oviposit on *P. tremuloides* and will not encounter *P. trifoliata* or *L. tulipifera* in its current range.

Therefore any loss or retention of host use abilities for these hosts will largely depend upon genetic drift.

Conclusion

Climate change has caused species ranges to shift significantly and is expected to continue to do so (e.g. Parmesan 1999, 2006), bringing formerly separated population ranges into closer contact. This situation has created a greater need to understand how gene flow between locally adapted populations of the same species or closely related species will affect the evolution of these organisms. This study illustrates how increased hybridization due to climate change can lead to significant rapid ecological divergence for traits that are not involved in reproductive isolation. We currently do not know whether the "late flight" hybrid swarm population will become completely reproductively isolated or not. However, the results presented here represent an instance where a shift in a major ecological trait, host use, is likely occurring as a byproduct of a shift in an unrelated trait leading to partial reproductive isolation. These changes in host use behavior include a Z(X)-linked trait (oviposition) present in a recombined Z(X)-chromosome and autosomally inherited non-additive larval detoxification abilities.

CHAPTER 5

GENERAL CONCLUSION

In this dissertation I have used the *P. glaucus* and *P. canadensis* system as a model system to understand how climate change is likely to affect the interaction between organisms. The results presented in Chapter 2 have indicated that while *P. canadensis* is significantly impacted by higher temperature regimes, occasional very hot summer temperatures do not appear to be the primary cause of the southern range limit of *P. canadensis*. This situation implies other factors are likely contributing to the range limit of *P. canadensis,* likely to include interactions with *P. glaucus.* In addition, the results presented in chapter 2 indicate that the expenditure of metabolic reserves, caused by increased temperatures, is likely to significantly reduce the fitness of *P. canadensis.* Therefore climate warming will not only increase movement of *P. glaucus* into *P. canadensis* territory, decreasing *P. canadensis* "mating success", but is also likely to decrease the survival of *P. canadensis* due to thermal stress.

The increased movement of *P. glaucus* into *P. canadensis* territory has already been observed in trait clines (Scriber 2002) and the formation of the "late flight" (Scriber and Ording 2005; Scriber *et al.*2008a). This situation has permitted the evaluation of how increased genetic introgression caused by climate change may alter plant-insect associations. As I noted in Chapter 3, the difference in ovipositional behavior of *P. canadensis* and *P. glaucus* is caused by a Z-linked trait for the acceptance of *P. tremuloides.* However, this small difference can have profound implications for the evolutionary trajectory of the plant-insect associations in this polyphagous group. In

particular, the results presented in Chapter 4 indicate that recombination leading to the "late flight" may also be leading to a host shift onto a secondary host of both parental species. This situation highlights our need to understand how gene flow between locally adapted populations of the same species or closely related species will affect the evolution of these organisms, and in general a need to understand how interactions between organisms will be affected by climate warming.

APPENDIX

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.: **2008-06**

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

SPECIES RANGES, HOST SELECTION, AND HYBRIDIZATION: HOW INCREASED HYBRIDIZATION IS LEADING TO HOST USE DIVERGENCE IN A POLYPHAGOUS SIBLING SPECIES PAIR.

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

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name(s) (typed)
Rodrigo Jose Mercader

Date 05/01/2008

*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

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 glaucus L.	Reared on Black Cherry (*Prunus serotina*) from females collected in Lancaster Co. Pennsylvania.					2	2		
Papilio canadensis R & J	Reared on Black Cherry (*Prunus serotina*) from females collected from the "early flight" (May/June) in the Battenkill River Valley NY/VT border.					2	2		
Papilio canadensis/ Papilio glaucus Hybrid Swarm	Reared on Black Cherry (*Prunus serotina*) from females collected from the "late flight" (Late July) in the Battenkill River Valley NY/VT border.					2	2		

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Rodrigo Jose Mercader

Date 5/1/2008

Voucher No. 2008-06

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

Curator Date 5 MAY 2008

Appendix 2

Abstracts/Summaries of closely related work on the ovipositional behaviour of the *P. glaucus* group not included in this dissertation.

Appendix 2.1

Summary for

Mercader RJ, Scriber JM (2005) Phenotypic plasticity of host selection in adult tiger swallowtails; Papilio glaucus (L.). In T.N. Ananthakrishnan and D. Whitman [ed.] Insects and phenotypic plasticity. pp. 25-57. Science Publishers, Enfield.

In summary, oviposition preferences of insects are determined by a large array of external and internal stimuli. Natural selection has acted on the genetic basis of oviposition ranking behavior, but phenotypic plasticity also plays a very important functional role. Behavioral preference induction in adults and larvae, as well as the effects of fecundity, age, and other factors may modify the 'acceptability threshold' for different hosts and determine how far down a ranking-order the insect will go (i.e. its 'specificity').

The eastern tiger swallowtail butterfly (*P. glaucus*) is the most polyphagous of all 560 species of Papilionidae (Scriber 1984a; 1995). Here we show that the ranking order of oviposition preference for this generalist appears basically constant in seven family host choice arrays for populations from the northernmost to southernmost parts of its geographic range (Fig 6). Local 'phenotypic differentiation' (as opposed to local 'adaptation') may be accomplished largely by phenotypically flexible (plastic) 'specificity' in host selection by individual females, thereby alleviating the directional selection for rank-order changes that might be expected in various local populations. The lack of local adaptations for changing rank-order of preference is not due to a lack of genetic variation in oviposition behavior; it is clear that individual females vary in their placement, with 100% of their eggs to less than 10% of their eggs on (the putatively ancestral) tulip tree favorite (Fig. 7). 'Plasticity' appears to dampen (lessen the need for) directional selection (that might take a ranking-order change to fixation). Ovipositional flexibility for local hosts or ecological conditions is thus (theoretically) possible without need for extensive gene flow because of the specificity adjustments in this *Papilio* species. In contrast, the avoidance of

aspen (both ranking order and specificity) may be genetically and behaviorally invariant, with almost every individual female placing few, if any eggs on this species (Fig 7).

The benefits of being polyphagous should exceed those of being specialized. The behavioral efficiency of locating the best host is generally less for generalists (perhaps due to neural limitations; Bernays 2001). Plasticity in behavior would be useful (e.g. where choices of hosts vary in availability in non-predictable fashion (as seen in generalists). Such non-genetic (inducible) responses may have permitted an ancient generalist (*P. glaucus*) to persist in ecologically and phytochemically diverse habitats trather than evolving numerous local host-specializations which would seem clearly possible, given the high levels of genetic variability for oviposition preferences that persists across the range of this generalist species (Bossart 2003, Bossart and Scriber 1995, 1999,; Fig. 7). Clearly, the complete ecology of the species needs to be addressed in order to understand the factors preserving this persistent polyphagy.

In addition to host plant and enemy-related factors, genetically-based behavioral oviposition divergence in time and location have been documented due to abiotic factors such as thermal selection as shown in highland/lowland *Drosophila* (Dahlgaard *et al.*2001) and in climatic 'cold pockets' for *Papilio canadensis* (Scriber 2002b). As seen here with *P. glaucus*, it may be that plasticity in specificity allows different genetically-determined rank orders among related *Papilio* species (Scriber *et al.* 2003), but usually not within *Papilio* species. For example, heritable variation has been shown to exist for *P. glaucus* toward less preferred hosts rather than ranking order (Bossart and Scriber 1999). Both ranking and specificity of butterfly oviposition may be largely X-linked and genetically-controlled (Janz 2003; Singer 2003). However, the role of phenotypic plasticity in determining oviposition variation observed between individuals, populations, and congeneric species will continue to be an important prerequisite for our understanding of host plant (and adult preference/larval performance correlations; Mayhew 2001; Bossart 2003) relationships in phtyophagous insects.

Appendix 2.2

Abstract for

Mercader RJ, Stelinski LL, Scriber JM (In Press). Differential antennal sensitivities of the generalist butterflies Papilio glaucus and Papilio canadensis to host plant and non-host plant extracts. Journal of Lepidopterists' Society

It is likely that olfaction is used by some generalist insect species as a pre-alighting cue to ameliorate the costs of foraging for suitable hosts. In which case, significantly higher antennal sensitivity would be expected to the volatiles of preferred over less or un-preferred host plants. To test this hypothesis, antennal sensitivity was measured by recording electroantennogram (EAG) responses from intact antennae of the generalists *Papilio glaucus* L. and *P. canadensis* R & J (Lepidoptera: Papilionidae) to methanolic leaf extracts of primary, secondary, and non-host plants. EAGs recorded from antennae of *P. glaucus* were approximately four fold higher than those of *P. canadensis* in response to extracts of its most suitable host plant, *Liriodendron tulipifera* (Magnoliaceae). Likewise, EAG responses of *P. canadensis* to its preferred host, *Populus tremuloides* (Salicaceae), were significantly higher than those of *P. glaucus*. In addition, *P. glaucus* exhibited significantly higher (approximately three fold) EAG responses to its preferred host, *L. tulipifera*, than to its less-preferred hosts, *Ptelea trifoliata*, *Sassafras albidum*, and *Lindera benzoin*. The results from this study indicate a significant divergence in the olfactory system of two closely related generalist butterfly species, including a strong specialization in the olfactory system of *P. glaucus*.

Appendix 2.3

Abstract for

Mercader RJ, Scriber JM (In Press) Divergence of ovipositional behavior in the *Papilio glaucus* group. Insect Science

This study contrasts the ovipositional profiles of 4 members of the *P. glaucus* group, *P. glaucus, P. multicaudatus, P. canadensis,* and *P. rutulus*. We use seven choice oviposition bioassays containing leaves from hosts in seven plant families utilized by members of the *P. glaucus* group. Specifically, we contrast the overall ovipositional profiles of these species and their acceptance of a host in a novel plant family (*Populus tremuloides*: Salicaceae) and a host in a putatively ancestral host plant family (*Liriodendron tulipifera*: Magnoliaceae). Significant differences were observed between the ovipositional profiles of *P. glaucus* and *P. multicaudatus* relative to each other and to *P. canadensis* and *P. rutulus.* In contrast, no significant differences were observed between the ovipositional profiles of *P. canadensis* and *P. rutulus,* which were also the only species that accepted *P. tremuloides*. Unlike the acceptance of *P. tremuloides*, the acceptance of *L. tulipifera* was present throughout the group despite the inability of the larvae of most species in the group to utilize this host. These results support the prediction of the "hierarchical threshold model" (Courtney *et al.,* 1989) that ancestral host plants are likely to be retained in the ovipositional hierarchy while novel hosts should only be accepted by derived populations.

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