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# LACK OF CRYPTIC REPRODUCTIVE ISOLATION BETWEEN *PAPILIO CANADENSIS* AND *PAPILIO GLAUCUS*; AND POPULATION GENETICS NEAR THEIR HYBRID ZONE

By

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ABSTRACT

LACK OF CRYPTIC REPRODUCTIVE ISOLATION BETWEEN *PAPILIO CANADENSIS* AND *PAPILIO GLAUCUS*; AND POPULATION GENETICS NEAR THEIR HYBRID ZONE

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The objectives of this thesis relate to the maintenance of species differences across a hybrid zone between the swallowtail butterflies *Papilio canadensis* and *Papilio glaucus*. The first objective was to determine if there is physiological (postpairing, prezygotic) isolation between these species. Heterospecific pairings between *canadensis* and *glaucus* were no less likely than conspecific pairings to last at least 30 minutes, result in spermatophore deposition, result in oviposition, or result in production of larvae. When females were mated to more than one male, there was no preferential use of sperm from conspecific males (conspecific sperm precedence). Together, these indicate that there is no physiological isolation between *canadensis* and *glaucus*.

The second general objective was to study gene flow, both within *canadensis* and between species. Allozymes indicate high gene flow between *canadensis* populations, with $F_{ST}$-values less than 0.01 for all four polymorphic enzyme loci used. Introgression of *glaucus* alleles into *canadensis* populations was found at two different types of loci: the X-linked nuclear *Pgd* gene, and in mitochondrial DNA. However, introgression was found only in *canadensis* populations nearest hybrid zone areas, indicating some genetic structure. When present, introgressed alleles were at low frequency, and individuals carrying introgressed alleles at one locus rarely carried introgressed alleles at other loci.

To my parents

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# INTRODUCTION

Closely related species with parapatric or sympatric distributions represent an interesting problem for biologists. Differences between them are generally maintained, even though they often still share significant similarity in their reproductive systems. This problem is especially interesting when two species meet at a hybrid zone, an area where they meet and interbreed (Barton & Hewitt 1985), and differences are maintained even in the face of hybrid production.

Such species allow the study of key questions in evolution and ecology. They represent an important stage of speciation: differentiation between the two groups, while limited enough to allow hybrid production, is complete enough to isolate the two (Hewitt 1988). They also allow the study of species boundaries, and why they lie where they do (Hoffman & Blows 1994).

This thesis addresses questions relating to the maintenance of species differences across a swallowtail butterfly hybrid zone. Because unhindered gene flow between two differentiated populations will quickly homogenize the two, in cases where species differences are maintained, there must be barriers to gene flow (Barton 1979). I examined potential barriers to gene flow that occur after mating begins but before hybrid zygote formation. Also, because in some cases gene flow between two species does not stop entirely (Barton & Hewitt 1985), I studied patterns of gene flow near and across the hybrid zone.

**Barriers to Gene Flow Between Species**

Darwin's (1859) consideration of the maintenance of species differences was limited to a discussion of the inviability and sterility of many hybrids. The Biological Species Concept (BSC), promoted by Dobzhansky (1951) and Mayr (1963), states that speciation has occurred when two groups of organisms are no longer capable of exchanging genes, and calls this state reproductive isolation. Both authors included classifications and examples of factors that can cause reproductive isolation in their treatments of the BSC.

The BSC has been criticized for a number of reasons (Mallet 1995, Harrison 1998), including a questioning of the need for a complete cessation of gene flow between recently diverged species. Many other species concepts have been proposed, but regardless of what concept individual researchers adhere to, the BSC has been important by focusing attention on factors that restrict gene flow between two species (Harrison 1998).

Mayr (1963) divided barriers to gene flow into two general categories: premating and postmating. Alternatively, these can be grouped as either prezygotic or postzygotic (Table 1.1), a more informative classification because broadly speaking, prezygotic barriers can potentially be selected for (Dobzhansky 1951), whereas postzygotic barriers cannot. This thesis will focus on physiological isolation between species: barriers to gene flow that occur after copulation has started, but before eggs are fertilized (Table 1.1). These barriers can be caused by divergence in genitalic physiology, by cryptic female choice (Eberhard 1996), or by competition between sperm from different males (Birkhead & Møller 1998).

Table 1.1. Barriers to gene flow between species (adapted from Campbell 1993).

| Category | Description |
|---|---|
| Prezygotic: | Prevents the production of hybrids |
| 1. Geographic Isolation | Species live in different areas |
| 2. Temporal Isolation | Species mate at different times |
| 3. Behavioral Isolation | Species meet, but do not attempt to mate |
| 4. Mechanical Isolation | Species attempt to copulate, but cannot |
| 5. Physiological Isolation* | Species copulate, but sperm does not reach egg |
| Postzygotic: | Reduces the fitness of hybrids |
| 1. Zygote Mortality | Eggs are fertilized but do not hatch |
| 2. Hybrid Inviability | Hybrid individuals die before sexual maturity |
| 3. Hybrid Sterility | Hybrids do not produce functional gametes |
| 4. Hybrid Breakdown | Offspring of hybrids have reduced viability or fertility |
| 5. Ecological Selection | Hybrids are poorly adapted to certain habitats |

* Refers to postpairing, prezygotic isolation, including: cryptic female choice, incapacitation of sperm, and conspecific sperm precedence.

*Physiological isolation with singly-mated female insects*

Differences in genitalia are often found between closely related insect species, and this observation has produced the 'lock-and-key' hypothesis: genitalic differences should mechanically prevent males from being able to inseminate females of other insect species (Dufour 1844). However, few examples have been found where the lock-and-key hypothesis holds (Dobzhansky 1951, Porter & Shapiro 1990). Recently though, other forms of postpairing[1], prezygotic isolation between species have been found.

Differences in genitalia may still play a part in species isolation, even when they do not mechanically prevent successful mating. Genitalic differences between species of scarab beetles appear to allow females to exercise choice about whether to allow full insemination by a male (Eberhard 1992). Courtship during copulation could have a

[1] For the purposes of this thesis, any factor that is referred to as postpairing will be something that occurs after a copulation has started (including events after copulation), and any factor called postcopulatory will refer to something occurring only after a copulation has ended.

similar effect (Eberhard 1994). In these cases, species differences are potential cues that females can take advantage of, leading to physiological isolation via female choice.

Physiological isolation can also be due to poor sperm performance. In some ladybird beetles, heterospecific crosses produce a lower percentage of hatching eggs than conspecific crosses (Nakano 1985), caused by a partial incapacitation of heterospecific sperm in the female's reproductive tract (Katakura 1986). In some ground crickets (Gregory & Howard 1993) and katydids (Shapiro 2000), females mated to conspecific males produce more eggs than those mated to heterospecific males, which could indicate conspecific sperm produces a stronger oviposition response.

Physiological isolation can also be asymmetric between species. In the cricket genus *Gryllus*, hybrid pairings between *G. firmus* females and *G. pennsylvanicus* males are unsuccessful, whereas pairings between *G. pennsylvanicus* females and *G. firmus* males are fully successful (Harrison 1983).

There can also be geographic variation in physiological isolation, as in two species of green lacewings with ranges that overlap in some areas and do not overlap in others (Albuquerque et al. 1996). When individuals taken from areas of sympatry are mated, heterospecific sperm fails to transfer to the spermatheca of the female. However, when individuals from areas where the other species is not found are mated, heterospecific sperm is transferred to the spermatheca and is used to fertilize eggs. This points toward reinforcement of premating isolation (Butlin 1989), although it should be remembered that these species share broad stretches of their ranges, allowing selection on many individuals. Where species meet at narrow hybrid zones, most individuals never

meet heterospecific individuals, so most individuals are never under any selection for reproductive isolation.

It is important to note that there may be many examples of insects where there is no postpairing, prezygotic isolation. In several species of longwing butterflies, heterospecific matings are just as successful as conspecific matings (McMillan et al. 1997), and there may be many other examples where this is true.

*Conspecific sperm precedence*

Parker (1970) introduced the idea that when insect females mate with multiple males and store sperm, sexual selection can continue even after copulation. This has usually been presented as sperm competition, where the sperm from one male competes with the sperm from other males to fertilize the eggs of the female (postcopulatory intrasexual selection) (Birkhead & Møller 1998). However, female choice, where a female chooses what sperm to use based on some characteristic of the males or their ejaculates (postcopulatory intersexual selection), may also play an important role (Eberhard 1996). Sperm precedence is the general term given to patterns of sperm usage by a female that has mated to more than one male (Simmons & Siva-Jothy 1998).

Different types of sperm precedence have been found in insects, with the most common being for the female to use a mixture of sperm from her various mates, biased to some degree towards the most recent male (Gwynne 1984). Mechanisms for this type of sperm precedence ('last-male') include the ejection of previous spermatophores (e.g. DeVilliers & Hanrahan 1991) or the repositioning or displacement of previous sperm (e.g. Siva-Jothy & Tsubaki 1994, Eady 1994). Another type is 'first-male' sperm

precedence, where all or almost all offspring continue to be sired by the first male, even after later copulations with one or more different males (Gwynne 1984). Large spermatophores acting as mating plugs can lead to this pattern (Lorch et al. 1998). There are also cases where a female will preferentially use sperm from a certain male based on some other factor (female choice), such as spermatophore size in arctiid moths (LaMunyon & Eisner 1994). It has been noted that there is often much variability in patterns of sperm usage from pairing to pairing (Simmons & Siva-Jothy 1998).

Most studies on sperm precedence have looked at postcopulatory sexual selection between individuals belonging to the same species. However, several cases have been found where sperm competition can result in a barrier to gene flow between species (Howard 1999). In ground crickets (Gregory & Howard 1994), flour beetles (Wade et al. 1994), a grasshopper (Hewitt et al. 1989), and some *Drosophila* (Price 1997), a female mated only to a heterospecific male will produce many hybrid offspring. However, when mated to a conspecific male and a heterospecific male, such a female will produce offspring sired almost exclusively by the conspecific male, regardless of the order of copulations. This is called conspecific sperm precedence, and it has been shown that when females mate multiply, it can be a potent barrier to gene flow between species (Gregory & Howard 1994, Howard et al. 1998).

Postcopulatory, prezygotic isolation has been called cryptic, because it is ignored by traditional Darwinian measures of success, which focus on male success in achieving mating access to females (Eberhard & Cordero 1995). However, physiological barriers could be important and under-appreciated in restricting gene flow between insect species.

## Population Genetics of Hybrid Zones

Hybrid zones are clines maintained by the opposing forces of dispersal and gene flow on the one hand, and reproductive isolation and selection against hybrids on the other hand (Barton & Hewitt 1985). This means that to understand how species differences are maintained, one must understand not just the barriers to gene flow, but also the potential of both species for dispersal and gene flow. This can be done either by studying gene flow within both species, or by studying gene flow between species (introgression).

### *Gene flow within species*

Gene flow is defined as the movement of genes between populations (Slatkin 1985). It is important because it tends to homogenize populations, counteracting drift and local adaptation, as well as spreading advantageous alleles (Slatkin 1987). If gene flow is high within two closely related species, the barriers to gene flow between them must be quite strong to maintain the differences between the two (Barton 1979). However, if gene flow within both species is low, the barriers between the two need not be as strong.

There are two basic approaches to studying gene flow within species: direct and indirect (Slatkin 1987). Direct measures of gene flow are based on observations of dispersing individuals. One weakness with this approach is that gene flow may be sporadic, and occasional events of high gene flow could be enough to homogenize populations significantly. Unless observations were made during these times, a direct approach would underestimate the effective gene flow that occurs over an evolutionary

time scale. The other basic approach is indirect: estimating gene flow by looking at the geographic patterns of allele and genotype distribution. Wright (1931) provided the earliest statistical tools for indirect measurement of gene flow, *F*-statistics. The development of allozyme electrophoresis provided many potentially neutral, codominant markers that are widely dispersed through the genome (Avise 1994), making them highly compatible with Wright's method.

Indirect methods of estimating gene flow based on allozymes have their limitations. One is that in some cases allozymes may provide a reflection of historical patterns of dispersal rather than current levels of gene flow (Bossart & Prowell 1998). However, historical patterns, if they reflect long-term potential for gene flow, might be more important to evolution than the pattern of relatively few recent years. Another limitation is that allozymes do not reveal much of the DNA variation that is present, even in the genes for the enzymes (Richardson et al. 1986). They may miss genetic discontinuities between populations that other markers may reveal (Karl & Avise 1992). However, while methods more powerful at detecting variation are continually being developed, allozymes remain the most accessible and cost-effective way of surveying genetic variation at many variable loci in a large number of individuals (Richardson et al. 1986).

*Interspecific introgression*

Introgression is a special case of gene flow: gene flow across species boundaries (Harrison 1993). There is debate about the importance of introgression to the evolution of parental species (Arnold et al. 1999), but in any case introgression can be informative

about the nature and completeness of barriers to gene flow between species. Hybrid zones are typically characterized by short, steep clines flanked by long tails of introgression on either side (Barton & Hewitt 1985). Measuring the length of tails, and the frequency of introgressed alleles in those tails, allows the calculation of the strength of selection against interspecific alleles as a function of distance from a hybrid zone (Porter et al. 1997).

Tails of introgression are rarely the same for all loci. In some groups it has been found that mitochondrial DNA introgresses more readily than nuclear genes (Barton & Jones 1983, Powell 1983), and selection may maintain differences in diagnostic traits while allowing significant gene exchange at other loci (Barton & Bengtsson 1986). Tails of introgression of mtDNA or enzyme loci may be longer than clines in quantitative traits such as morphological characters (Barton & Hewitt 1985), or narrower than other quantitative traits such as host use abilities (Hagen 1990). The width of clines and the length of tails of introgression should reflect the strength of selection against these introgressed characters.

If introgression varies at different geographic points along a hybrid zone, or if it changes through time, it could be informative about what is causing species boundaries and barriers to gene flow. For example, abnormally warm years might allow increased introgression of genes from southern species into closely related neighboring northern species. Introgression might also be asymmetric between species (Sperling & Spence 1991).

The genotypic pattern that introgression takes is important as well. When it is present, whether it is found at homozygous or heterozygous loci, and whether

introgression at one locus tends to be coincidental within individuals with introgression at other loci can indicate how recent the gene flow across the hybrid zone was.

**Isolation in Tiger Swallowtails: *Papilio canadensis* and *Papilio glaucus***

The Eastern Tiger Swallowtail, *Papilio glaucus* L., and the Canadian Tiger Swallowtail *P. canadensis* Rothschild & Jordan (Lepidoptera: Papilionidae) are closely related butterfly species with parapatric distributions (Figure 1.1). Until fairly recently, *canadensis* was considered to be a subspecies of *glaucus*, but based on morphological, molecular, and ecological differences (Table 1.2), it was given separate species status (Hagen et al. 1991). Several of the differences follow a pattern in Lepidoptera: a disproportionately high number of diagnostic traits (*Ldh*, *Pgd*, diapause induction, dark color suppression, Hagen & Scriber 1989) are X-linked (Sperling 1994).

Allopatric speciation has been hypothesized for *canadensis* and *glaucus*: speciation during the Pleistocene ice age, with the proto-*glaucus* populations spending the last 40,000 years south of the glaciation and the proto-*canadensis* populations isolated in the unglaciated pocket of Beringia, now Alaska (Scriber 1988). However, there is higher variability in allozymes in Michigan *canadensis* populations than in Alaskan *canadensis* populations (Hagen & Scriber 1991). Because ancestral populations often are more genetically variable than dispersed populations, one could hypothesize parapatric speciation, although population size or introgression could also account for the differences seen.

Both *canadensis* and *glaucus* are found over wide geographic ranges (Figure 1.1), covering a number of different ecological habitats. Local adaptation to regional habitats

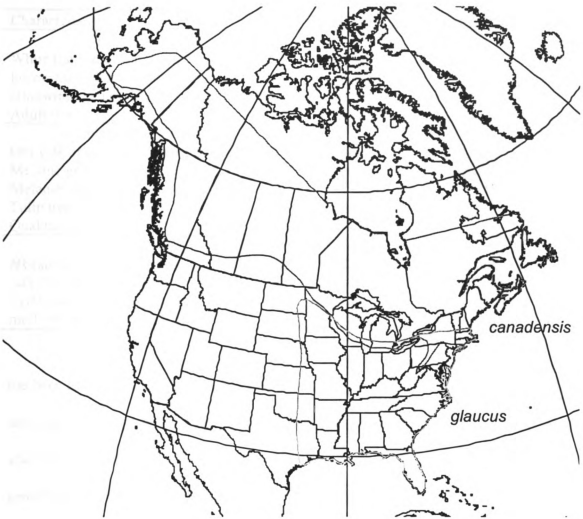


Figure 1.1. Ranges of *canadensis* and *glaucus* (adapted from Hagen & Scriber 1991).

Table 1.2. Species differences between *canadensis* and *glaucus* (Hagen et al. 1991).

| Characteristic | *canadensis* | *glaucus* |
|---|---|---|
| (Morphological) | | |
| White transverse bands on 1st instar larvae | 3 | 1 |
| Forewing underside submarginal yellow | Band | Spots |
| Hindwing upperside anal cell black band | Wide | Narrow |
| Adult size | Small | Large |
| (Ecological/Physiological) | | |
| Obligate diapause (X-linked recessive) | Present | Absent |
| Melanic gene (Y-linked) | Absent | Present |
| Melanic suppressor gene (X-linked) | Present | Absent |
| Tulip tree use ability | Low | High |
| Quaking aspen use ability | High | Low |
| (Molecular) | | |
| *Hk* (autosomal) allozymes | HK 110 | HK 100 |
| *Ldh* (X-linked) allozymes | LDH 40, 80 | LDH 100 |
| *Pgd* (X-linked) allozymes | PGD -80, -125 | PGD -50, -100 |
| mtDNA *Taq*I site in *COI* gene | Absent | Present |

has been found in both species: *canadensis* to thermal climates (Ayres & Scriber 1994), and *glaucus* to regional hostplants (Scriber 1986, Bossart & Scriber 1995). Local adaptation in *glaucus* contrasts with a finding, based on allozyme distributions, of high gene flow between widely separated populations (Bossart & Scriber 1995) and evidence of high dispersal potential of *glaucus* individuals (Lederhouse 1982, Scriber et al. 1998).

The ranges of *canadensis* and *glaucus* meet at a narrow hybrid zone (Hagen et al. 1991, Hagen 1990, Luebke et al. 1988). Study of the hybrid zone in New York using allozymes found no evidence of assortative mating in the zone (Hagen 1990). F1 hybrids are viable, fertile (Hagen & Scriber 1995), and able to survive on the hostplants of either parental species (Scriber et al. 1995). Long-range dispersal of a hybrid out of the hybrid zone has been documented (Scriber et al. 1998). One focus of research on these *Papilio* species is to determine what keeps them distinct.

A couple of potential barriers to gene flow have been found. Deering (1998) found behavioral isolation between the two species, with a caveat. In Florida, *glaucus* males choose to court and copulate with *glaucus* females rather than equally sized *canadensis* females. However, *canadensis* males in northern Michigan also prefer *glaucus* females over *canadensis*. In a hybrid zone, the preference of *glaucus* males for conspecific females would reduce hybridization, but the preference of *canadensis* males for heterospecific females would increase hybridization.

A common pattern in species hybridization is the Haldane Effect: hybrids of the heterogametic sex (females in the Lepidoptera) often have lower fitness than hybrids of the homogametic sex (Coyne & Orr 1989). A Haldane effect is seen in one of the types of crosses between *canadensis* and *glaucus* (Hagen & Scriber 1995). When a *glaucus* female is paired to a *canadensis* male, female offspring have higher pupal mortality than males, but when a *canadensis* female is paired to a *glaucus* male, there is no increase in pupal mortaility in either sex. This Haldane effect is apparently due to *canadensis* X-linked genes, when combined with *glaucus* genes, disrupting pupal development (Hagen & Scriber 1995). Slight endogenous reduction of hybrid fitness such as this could be expected to reduce gene flow between the species, but would not prevent it. The search is still on for barriers to gene flow between *canadensis* and *glaucus*.

A possible barrier to gene flow might be postpairing, prezygotic isolation. These butterflies might be good candidates for such isolation for two reasons: 1) females mate more than once (Lederhouse et al. 1989), so even if a female copulates with the wrong kind of male, her reproductive potential can be rescued by finding a better male; and 2) sperm are passed in large, possibly nutritious spermatophores (Lederhouse 1995), which

could act as a cues to females about the quality and appropriateness of males. Postcopulatory isolation might also be facilitated by the female reproductive system of butterflies. Butterfly females, as in most of the Lepidoptera, are ditrysian, meaning they have two genital openings: one for copulation, one for oviposition (Figure 1.2). When mating, a male secretes a spermatophore, sperm, and other ejaculate into the bursa copulatrix. Sperm then leaves the spermatophore and travels through the ductus seminalis to the spermatheca. Sperm from the spermatheca is then used to fertilize eggs as they pass through the oviduct on the way to be oviposited. This physiology may make it more likely for heterospecific sperm to be incompatible with the female reproductive tract, or it may allow the female increased postcopulatory choice (Tschudi-Rein & Benz 1990). Postpairing, prezygotic isolation could appear as a reduction of success of heterospecific pairings relative to conspecific pairings, or as conspecific sperm precedence.

Another focus of research on *Papilio* is their population genetics. Gene flow has already been studied in *glaucus* populations (Bossart & Scriber 1995), however there is

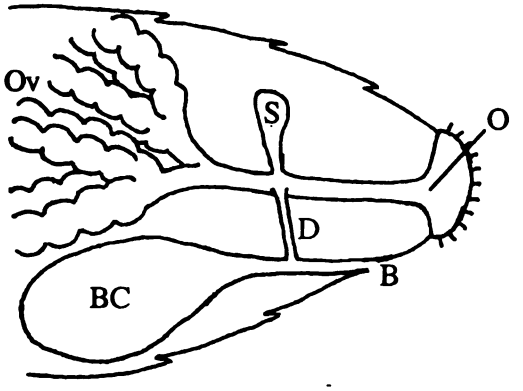


Figure 1.2. Schematic of female genitalia of ditrysian Lepidoptera; lateral cross section of posterior half of abdomen. BC: bursa copulatrix; S: spermatheca; O: ostium oviductus; B: ostium bursae; D: ductus seminalis; Ov: ovaries. Modified from Drummond (1984).

no companion study for *canadensis*. Also, introgression of diagnostic allozymes has been documented (Hagen et al. 1991), but introgression of maternally inherited mitochondrial genes has not yet been investigated. Previous work with these species has created a supply of genetic markers ready to be used to continue the study of their population genetics (Hagen & Scriber 1991, Sperling 1994).

**Objectives**

My objectives for these studies were: 1) to determine if heterospecific pairings between *canadensis* and *glaucus* are less successful than conspecific pairings; 2) to determine if there is conspecific sperm precedence in either *canadensis* or *glaucus*; 3) to indirectly determine levels of gene flow between isolated *canadensis* populations using allozyme electrophoresis; and 4) to determine levels of interspecific introgression of *glaucus* genes into *canadensis* populations using both allozymes (for nuclear introgression) and PCR-RFLP (for mitochondrial introgression).

## CHAPTER 2:

## ARE HETEROSPECIFIC PAIRINGS BETWEEN *PAPILIO CANADENSIS* AND *P. GLAUCUS* LESS SUCCESSFUL THAN CONSPECIFIC PAIRINGS?

### Introduction

Hybrid zones, geographic areas where individuals of different species meet and interbreed, are not uncommon in nature (Barton & Hewitt 1985). Generally, the distinctness between the species at large is still maintained, even in the face of hybrid production. Even where hybrids are found, if heterospecific matings are less successful[1] than conspecific matings, fewer hybrids will be produced and gene flow will be reduced between species. This type of postpairing, prezygotic barrier to gene flow could arise either from divergent reproductive physiologies or from cryptic mate choice (Eberhard 1996).

Hybrids with high viability and fertility are common in *Papilio*, and it has been suggested that because of this, prezygotic barriers between species should be important (Sperling 1990). These could include postpairing barriers in addition to premating isolation. Lab matings between *Papilio canadensis* and *P. glaucus* produce viable and fertile hybrids (Scriber et al. 1995), but it is not known if heterospecific pairings are less successful than conspecific pairings.

The complicated reproductive tract of females of the ditrysian Lepidoptera means that there are a number of stages at which a mating can fail. Hand-paired *Papilio* butterflies will often struggle against each other immediately after being paired, and at

[1] Mating success will refer to a number of factors: pairing duration, spermatophore deposition, oviposition, and egg hatchability. Of course, the final measure of mating success is production of offspring.

this point they can break apart easily. This may be a response to their mate, or it could be a response to significant human handling. As Clarke and Sheppard (1956) observed, after several minutes the two seem to lock together and some pulling will not separate them. Coincident with this locking, the head and thorax of the male relax, and both individuals become still. At this point, pairs typically remain together for upwards of half an hour. Lab pairings with *glaucus* have found that pairings must last at least 30 minutes (at least in the lab) for the male to transfer a spermatophore (Lederhouse et al. 1990). Thus a pairing can be unsuccessful due to breaking up prematurely either before locking occurs (in the first few minutes) or after locking (the pairing lasts more than a few minutes, but fewer than 30 minutes). The first is best studied in conditions as natural as possible to minimize handling effects, but the second could legitimately be studied using hand-pairings in lab conditions.

Even if a pairing lasts the minimum amount of time, it is not successful if it does not result in spermatophore deposition or if it fails to spur the female to lay eggs. Even if these successfully occur, if sperm is not moved to the spermatheca in significant numbers, females may lay only unfertilized eggs or a low percentage of fertilized eggs. Heterospecific pairings may fail more frequently than conspecific pairings at any of these points.

I investigated postpairing, prezygotic reproductive isolation between *Papilio canadensis* and *P. glaucus*. First, I asked some basic questions relating to mating success: 1) does the duration of a copulation affect its chance of being successful; and 2) can a mating be successful without spermatophore deposition? Second, I used *canadensis* and *glaucus* to compare heterospecific mating success to conspecific mating

success with respect to copulation duration, spermatophore deposition, oviposition, and production of larvae. Third, I investigated the effect of increased phylogenetic distance on mating success by pairing females of the more distantly related species *Papilio troilus* to *glaucus* males. Finally, I investigated the success of matings involving a type of interspecific hybrid by pairing *canadensis* × *glaucus* hybrid females to *canadensis* males.

**Methods**

Lab-reared, virgin females were used for all pairings. Males had either been reared in the lab or caught in the wild. Females were fed a 20% honey in water solution. Males were fed a 20% honey solution containing electrolytes and amino acids to increase virility (Lederhouse et al. 1990). Lab-reared males were not paired for at least two days following adult eclosion to allow reproductive maturation.

All matings were initiated by hand (Clarke & Sheppard 1956), and pairing duration was recorded. All pairings breaking apart before the individuals locked together (usually about five minutes in) were either re-paired or disregarded. After pairing, females were placed in plastic oviposition arenas lined with hostplant foliage following Scriber (1993) to stimulate oviposition, an established technique for facilitating egg production in our lab. Eggs were counted daily and placed in a growth chamber. Hatching larvae were also counted daily. Dead females were stored in a freezer, and later dissected to determine if a spermatophore was present (Lederhouse et al. 1989). For various reasons, not all data were collected for some pairings.

To determine the minimum parameters for a successful mating, I combined pairings of various types (conspecific, heterospecific, backcross, and $F_2$), involving

various *Papilio* species (*canadensis*, *glaucus*, *eurymedon*, *rutulus*, *multicaudatus*, and *troilus*), and using lab-reared females and lab-reared or wild-caught males, into several general comparisons. I compared pairings of different durations with respect to likelihood to lead to spermatophore deposition, oviposition, and production of hatching larvae. I also compared pairings where spermatophore deposition had occurred to those where it had not with respect to likelihood to lead to oviposition and larval production. These proportions were compared using a chi-square analysis (PROC FREQ; SAS Institute Inc. 1990). The proportion of pairings leading to larvae, out of all those leading to oviposition, was also calculated.

To compare the success of conspecific and heterospecific pairings, I paired lab-reared female *canadensis* and *glaucus* to lab-reared and wild-caught male *canadensis* and *glaucus*. For each of the eight pairing types, I calculated: the proportion of copulations lasting at least 30 minutes, out of all pairings reaching a locked state; the proportion of copulations resulting in spermatophore deposition, out of all pairings that lasted at least 30 minutes; the proportion of pairings leading to the female laying at least one egg, out of all pairings that resulted in a spermatophore being deposited; and the proportion of pairings leading to at least one hatching larva, out of all pairings that had led to oviposition. The effects of female and male species (*canadensis* or *glaucus*) and male origin (lab-reared or wild-caught) on these measures were determined using a contingency table analysis (PROC CATMOD; SAS Institute Inc. 1990). For each pairing resulting in at least one hatching larva, the number of larvae was divided by the number of eggs laid to give the proportion of hatching eggs. Then these proportions were averaged for each of the eight pairing types, to give average egg hatchability. The effects

of female and male species and male origin on egg hatchability were determined using an analysis of variance (PROC GLM; SAS Institute Inc. 1990).

To determine the effect of increased phylogenetic distance on heterospecific mating success, pairings between wild *glaucus* males and *glaucus* and *canadensis* females were compared to pairings between wild *glaucus* males and females of the more phylogenetically distant *Papilio troilus* (Hagen & Scriber 1991, Caterino & Sperling 1999). I also paired *canadensis* × *glaucus* hybrid females to wild *canadensis* males and compared them to pairings between wild *canadensis* males and *canadensis* and *glaucus* females to determine if pairings with a type of hybrid female show reduced success. Pairing success for these comparisons was analyzed as above, except instead of looking at species effects, the pairing types were simply compared.

Because the preceding comparisons break matings down into individual components, they do not provide an overall picture of reproductive success. For this, all pairings for which the first four measures were known (pairing duration, spermatophore deposition, whether eggs had been laid, and whether larvae had been produced) were compiled. The numbers successful in each measure were compared for each pairing type, and the proportion of all of these pairings that produced larvae was calculated for each pairing type. Then, to combine this with egg hatchability data, that proportion of pairings that produced any larvae was multiplied by the average egg hatchability for each pairing type. This gave an overall index of mating success that could be compared for each of the types of pairings.

**Results**

No pairings lasting for fewer than 30 minutes resulted in spermatophore deposition (Table 2.1). About half of the pairings 30-39 minutes long produced spermatophores, and almost all pairings lasting 40 minutes or longer resulted in spermatophores.

Some pairings from all duration divisions led to female oviposition (Table 2.1). Pairings lasting for fewer than twenty minutes were less likely to stimulate oviposition, but those only 20-29 minutes long produced females that were just as likely to oviposit as those copulating for longer.

Of pairings that resulted in oviposition, none lasting for fewer than 30 minutes resulted in hatching larvae, but most of those lasting 30 minutes or longer did result in larvae (Table 2.1). Thus while pairings lasting for fewer than 30 minutes can spur a female to oviposit, they do not result in spermatophore deposition or larval production. It appears that the minimum time for a completely successful mating is 30 minutes, at least at room temperature (75-85 F°).

Pairings resulting in spermatophore deposition were only marginally more likely to result in oviposition than those not leading to spermatophores (Table 2.2). However, pairings resulting in spermatophore deposition were significantly more likely to lead to hatching larvae after oviposition than those not. Out of 24 pairings without spermatophores that led to oviposition, two led to hatching larvae. Thus females can easily be induced to lay eggs without being provided a spermatophore, however larval production with no spermatophore (while possible) is rare. This result of larval production without a spermatophore is surprising, but it has been observed previously in

Table 2.1. Success of pairings grouped by mating duration. Proportions ± s.d.[1] are followed by sample sizes in parentheses. The pairings were of various types (conspecific, heterospecific, backcross, and $F_2$), involved various *Papilio* species (*canadensis*, *glaucus*, *eurymedon*, *rutulus*, *multicaudatus*, and *troilus*), and were between lab-reared females and either lab-reared or wild-caught males.

| Duration (minutes) | Proportion of pairings with a spermatophore | | Proportion of pairings with eggs | | Proportion of pairings with larvae (of those with eggs) | |
|---|---|---|---|---|---|---|
| 5-19 | 0.00 ± 0.00 | (4) | 0.33 ± 0.19 | (6) | 0.00 ± 0.00 | (2) |
| 20-29 | 0.00 ± 0.00 | (6) | 0.75 ± 0.12 | (12) | 0.00 ± 0.00 | (9) |
| 30-39 | 0.59 ± 0.12 | (17) | 0.75 ± 0.09 | (24) | 0.41 ± 0.12 | (17) |
| 40-49 | 0.94 ± 0.03 | (54) | 0.78 ± 0.05 | (70) | 0.66 ± 0.06 | (53) |
| 50-59 | 0.98 ± 0.02 | (62) | 0.77 ± 0.05 | (73) | 0.64 ± 0.07 | (47) |
| 60-69 | 1.00 ± 0.00 | (54) | 0.73 ± 0.05 | (74) | 0.51 ± 0.07 | (53) |
| 70-79 | 1.00 ± 0.00 | (19) | 0.70 ± 0.08 | (30) | 0.60 ± 0.11 | (20) |
| 80-89 | 0.94 ± 0.06 | (16) | 0.76 ± 0.08 | (25) | 0.61 ± 0.11 | (18) |
| 90-99 | 0.62 ± 0.17 | (8) | 0.69 ± 0.12 | (16) | 0.38 ± 0.17 | (8) |
| 100-109 | 0.78 ± 0.14 | (9) | 0.73 ± 0.13 | (11) | 0.38 ± 0.17 | (8) |
| 110-119 | 1.00 ± 0.00 | (4) | 0.83 ± 0.15 | (6) | 0.60 ± 0.22 | (5) |
| 120-129 | 1.00 ± 0.00 | (4) | 1.00 ± 0.00 | (7) | 0.57 ± 0.19 | (7) |
| ≥130 | 0.60 ± 0.22 | (5) | 0.86 ± 0.13 | (7) | 0.00 ± 0.00 | (5) |

[1] s.d.=√(proportion×(1-proportion)/sample size)

Table 2.2. Success of pairings grouped by whether a spermatophore had been deposited or not. Proportions ± s.d.[1] are followed by sample sizes in parentheses. Chi-square values compare the success of pairings resulting in spermatophore deposition with those not leaving spermatophores. The pairings were of various types (conspecific, heterospecific, backcross, and $F_2$), involved various *Papilio* species (*canadensis*, *glaucus*, *eurymedon*, *rutulus*, *multicaudatus*, and *troilus*), and were between lab-reared females and either lab-reared or wild-caught males.

| | Proportion of pairings with eggs | | Proportion of pairings with larvae (of those with eggs) | |
|---|---|---|---|---|
| Spermatophore absent | 0.62 ± 0.07 | (45) | 0.08 ± 0.06 | (24) |
| Spermatophore present | 0.75 ± 0.03 | (274) | 0.57 ± 0.04 | (189) |
| | df=1, $\chi^2$=3.322# | | df=1, $\chi^2$=19.867**** | |

# $P \leq 0.10$; **** $P \leq 0.001$

[1] s.d.=√(proportion×(1-proportion)/sample size)

*Papilio* (Lederhouse et al. 1989) and other insects (George & Howard 1968), and could be due to deposition of free sperm by the male (which seems more likely than parthenogenesis by the female). There was another surprising result: one singly-mated female was carrying two spermatophores, which has also been seen previously in *Papilio* (Lederhouse et al. 1989). Of all laboratory hand-pairings leading to oviposition, 0.54 ± 0.03 (proportion ± s.d.) led to hatching larvae (N=339).

The proportions of "locked-together" pairings (lasting for longer than about five minutes) involving *canadensis* and *glaucus* females and males that lasted at least the minimum 30 minutes are shown in Figure 2.1. Almost all of these pairings lasted at least 30 minutes, and there were no significant differences between pairing types. There were no significant effects on pairing duration (Table 2.3).

The proportions of pairings resulting in spermatophore deposition, out of all pairings lasting at least 30 minutes are shown in Figure 2.2. There were no significant differences between most pairing types. Lab-reared *canadensis* males were slightly less effective in depositing a spermatophore when mated to *glaucus* females, but there was no reduction in success when the *canadensis* male was from the wild. There were no significant effects on spermatophore deposition (Table 2.4).

The proportions of pairings leading to oviposition, out of all pairings resulting in spermatophore deposition are shown in Figure 2.3. There were again no significant differences between most pairing types. There was a significant effect of the species of the female, with *canadensis* females less likely to oviposit than *glaucus* females (Table

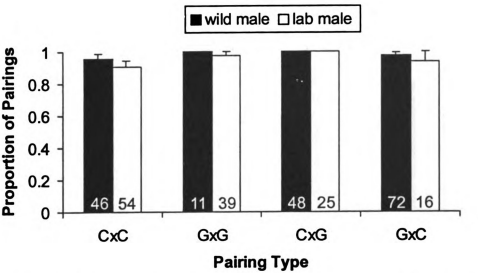


**Figure 2.1. Out of all pairings that lasted a minimum of five minutes, the proportion lasting at least 30 minutes. Error bars are +1 s.d. and numbers in bars are sample sizes. Female *canadensis* (C) or *glaucus* (G) were paired to male *canadensis* or *glaucus*, with the female listed first. Filled bars indicate that the males used were wild-caught, open bars indicate that the males used were lab-reared.**

Table 2.3. Chi-square values from ANOVA of the proportions of pairings involving *canadensis* and *glaucus* males and females lasting at least 30 minutes, out of all pairings that lasted a minimum of five minutes. The model was a 2×2×2 factorial design with effects being the species of the male, the species of the female (*canadensis* or *glaucus* for each), the origin of the male (wild or lab), and their interactions.

| Source of variation | df | $\chi^2$ |
|---|---|---|
| female species | 1 | 0.00 |
| male species | 1 | –[1] |
| female species*male species | 1 | 0.01 |
| male origin | 1 | 0.00 |
| female species*male origin | 1 | 0.00 |
| male species*male origin | 1 | 0.00 |
| female species*male species*male origin | 1 | 0.00 |

[1] $\chi^2$ not calculated (df=0) due to near fixation of success in pairings involving *glaucus* males.

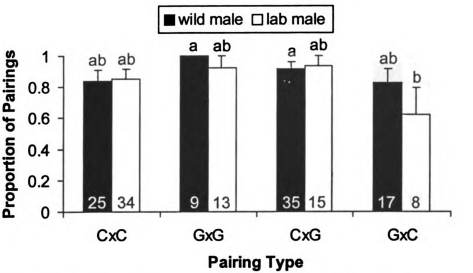


Figure 2.2. Out of all pairings lasting at least 30 minutes, the proportion resulting in spermatophore deposition. Error bars are +1 s.d. and numbers in bars are sample sizes. Bars not sharing a letter are significantly different from each other at $p$=0.05. Female *canadensis* (C) or *glaucus* (G) were paired to male *canadensis* or *glaucus*, with the female listed first. Filled bars indicate that the males used were wild-caught, open bars indicate the the males used were lab-reared.

Table 2.4. Chi-square values from ANOVA of proportions of pairings involving *canadensis* and *glaucus* males and females resulting in spermatophore deposition, out of all pairings lasting at least 30 minutes. The model was a 2×2×2 factorial design with effects being the species of the male, the species of the female (*canadensis* or *glaucus* for each), the origin of the male (wild or lab), and their interactions.

| Source of variation | df | $\chi^2$ |
|---|---|---|
| female species | 1 | 0.00 |
| male species | 1 | 0.00 |
| female species*male species | 1 | 0.00 |
| male origin | 1 | 0.00 |
| female species*male origin | 1 | 0.00 |
| male species*male origin | 1 | 0.00 |
| female species*male species*male origin | 1 | 0.00 |


Figure 2.3. Out of all pairings with spermatophore deposition, the proportion leading to oviposition. Error bars are +1 s.d. and numbers in bars are sample sizes. Bars not sharing a letter are significantly different from each other at $p$=0.05. Female *canadensis* (C) or *glaucus* (G) were paired to male *canadensis* or *glaucus*, with the female listed first. Filled bars indicate the males used were wild-caught, open bars indicate the males used were lab-reared.

Table 2.5. Chi-square values from ANOVA of proportions of pairings involving *canadensis* and *glaucus* males and females leading to oviposition, out of all pairings with spermatophore deposition. The model was a 2×2×2 factorial design with effects being the species of the male, the species of the female (*canadensis* or *glaucus* for each), the origin of the male (wild or lab), and their interactions.

| Source of variation | df | $\chi^2$ |
|---|---|---|
| female species | 1 | 3.85* |
| male species | 1 | 0.31 |
| female species*male species | 1 | 0.00 |
| male origin | 1 | 0.31 |
| female species*male origin | 1 | 0.04 |
| male species*male origin | 1 | 0.93 |
| female species*male species*male origin | 1 | 2.93# |

# $P \leq 0.10$; * $P \leq 0.05$

2.5). There was also a marginally significant three-way interaction between male species, female species, and male origin.

The proportions of pairings leading to production of larvae, of those leading to oviposition are shown in Figure 2.4. There was a significant effect of male origin (wild-caught males were more successful than lab-reared), and there was a significant interaction between male species and male origin (lab-reared *canadensis* males were less successful than wild-caught *canadensis* males, whereas lab-reared *glaucus* males were equivalent to wild-caught *glaucus* males) (Table 2.6). There was also a significant interaction between female species and male species, with heterospecific pairings slightly more likely to produce larvae than conspecific pairings.

The average egg hatchabilities of clutches containing at least one hatching egg are shown in Figure 2.5. There was a significant effect of male species (*glaucus* males led to greater average egg hatchability than *canadensis* males) and a significant effect of male origin (wild-caught males led to greater average egg hatchability than lab-reared males) (Table 2.7).

Thus in pairings involving *canadensis* and *glaucus* males and females, only one component of mating success (proportion of pairings producing larvae, out of all leading to oviposition; Figure 2.4) showed a significant difference between heterospecific and conspecific success (as shown by a significant female species*male species interaction; Table 2.6). However, heterospecific pairings were more likely to produce larvae than conspecific pairings. None of these components of mating success had heterospecific pairings significantly less successful than conspecific pairings.

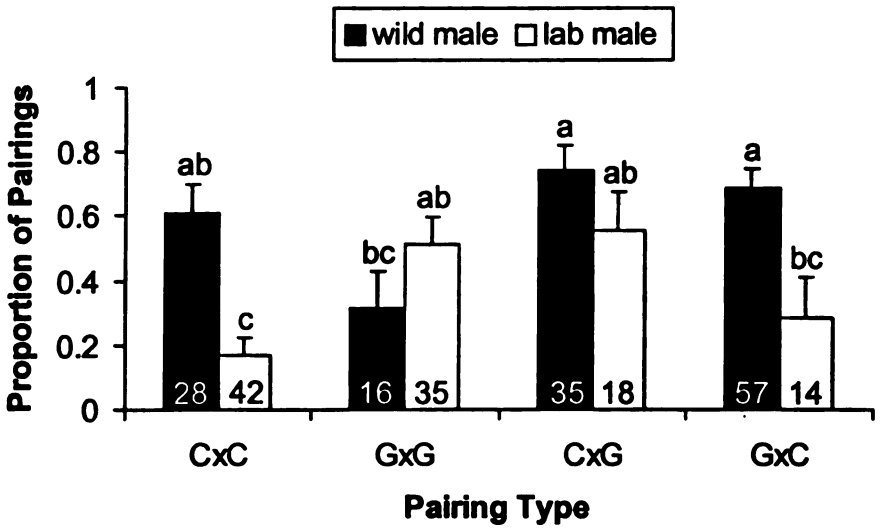


**Figure 2.4. Out of all pairings leading to oviposition, the proportion leading to production of larvae. Error bars are +1 s.d. and numbers in bars are sample sizes. Bars not sharing a letter are significantly different from each other at $p$=0.05. Female *canadensis* (C) or *glaucus* (G) were paired to male *canadensis* or *glaucus*, with the female listed first. Filled bars indicate the males used were wild-caught, open bars indicate the males used were lab-reared.**

Table 2.6. Chi-square values from ANOVA of proportions of pairings involving *canadensis* and *glaucus* males and females leading to production of larvae, out of all pairings leading to oviposition. The model was a 2×2×2 factorial design with effects being the species of the male, the species of the female (*canadensis* or *glaucus* for each), the origin of the male (wild or lab), and their interactions.

| Source of variation | df | $\chi^2$ |
|---|---|---|
| female species | 1 | 0.63 |
| male species | 1 | 2.28 |
| female species*male species | 1 | 6.06* |
| male origin | 1 | 9.06*** |
| female species*male origin | 1 | 2.71# |
| male species*male origin | 1 | 9.14*** |
| female species*male species*male origin | 1 | 1.15 |

# $P \leq 0.10$; * $P \leq 0.05$; *** $P \leq 0.005$

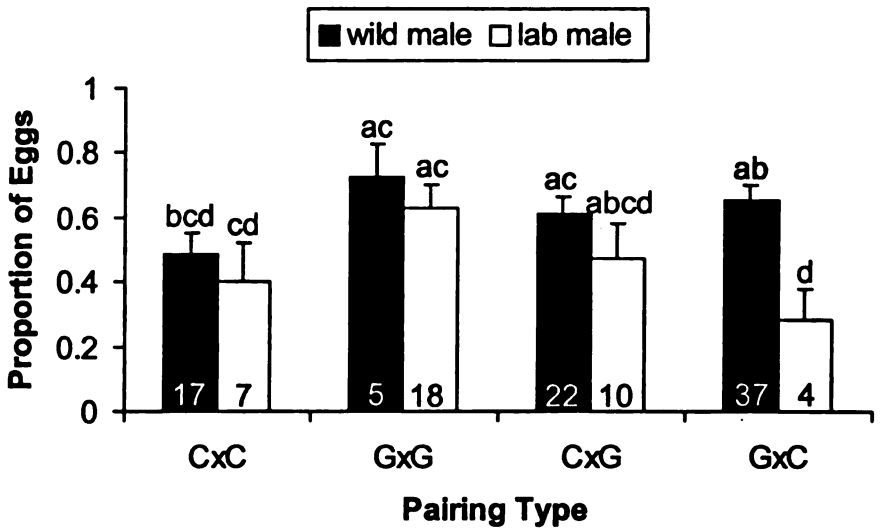


Figure 2.5. For all pairings producing at least one hatching larva, the mean proportion of viable eggs (hatching/total eggs). Values expressed are means, error bars are +1 s.e. and numbers in bars are numbers of pairings. Bars not sharing a letter are significantly different from each other at $p$=0.05. Female *canadensis* (C) or *glaucus* (G) were paired to male *canadensis* or *glaucus*, with the female listed first. Filled bars indicate the males used were wild-caught, and open bars indicate the males used were lab-reared.

Table 2.7. F-values (Type III SS) from ANOVA of proportions of hatching eggs out of all eggs laid, averaged over all pairings involving *canadensis* and *glaucus* males and females producing at least one hatching larva. The model was a 2×2×2 factorial design with effects being the species of the male, the species of the female (*canadensis* or *glaucus* for each), the origin of the male (wild or lab), and their interactions.

| Source of variation | df | F |
|---|---|---|
| female species | 1 | 1.48 |
| male species | 1 | 5.38* |
| female species*male species | 1 | 0.73 |
| male origin | 1 | 6.86** |
| female species*male origin | 1 | 0.89 |
| male species*male origin | 1 | 0.76 |
| female species*male species*male origin | 1 | 1.52 |

* $P \leq 0.05$; ** $P \leq 0.01$

When comparing *troilus* × *glaucus* pairings to *glaucus* × *glaucus* and *canadensis* × *glaucus* pairings, all pairings that locked together lasted at least 30 minutes. There were also no significant differences in proportions of pairings resulting in spermatophore deposition (Figure 2.6) or leading to oviposition (Figure 2.7). Out of all pairings leading to oviposition, the two types of heterospecific pairings were significantly more likely to lead to production of larvae than were the conspecific pairings (Figure 2.8). The *troilus* × *glaucus* pairings led to a lower average egg hatchability than the other two types of pairings (*canadensis* × *glaucus* and *glaucus* × *glaucus*) (Figure 2.9).

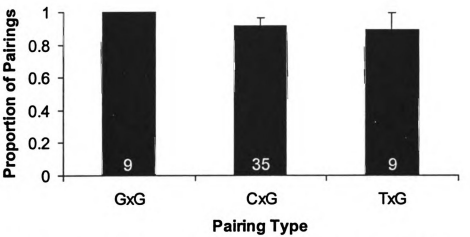


Figure 2.6. Out of all pairings lasting at least 30 minutes, the proportion of pairings resulting in spermatophore deposition. Error bars are +1 s.d. and numbers in bars are sample sizes. Female *glaucus* (G), *canadensis* (C), or *troilus* (T) were paired to wild-caught male *glaucus*, with the female listed first.

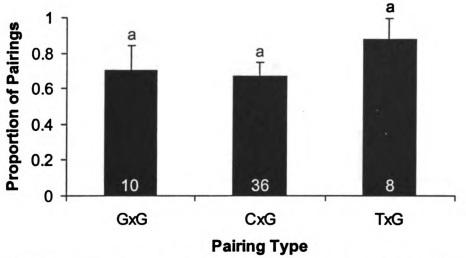


Figure 2.7. Out of all pairings with spermatophore deposition, the proportion leading to oviposition. Error bars are +1 s.d. and numbers in bars are sample sizes. Bars not sharing a letter are significantly different from each other at $p$=0.05. Female *glaucus* (G), *canadensis* (C), or *troilus* (T) were paired to wild-caught male *glaucus*, with the female listed first.

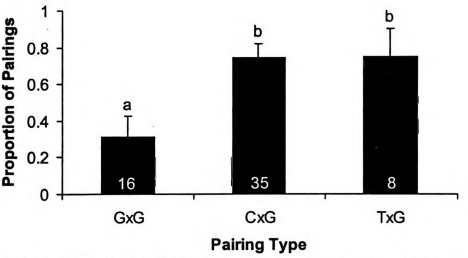


Figure 2.8. Out of all pairings leading to oviposition, the proportion of pairings leading to production of larvae. Error bars are +1 s.d. and numbers in bars are sample sizes. Bars not sharing a letter are significantly different from each other at $p$=0.05. Female *glaucus* (G), *canadensis* (C), or *troilus* (T) were paired to wild-caught male *glaucus*, with the female listed first.

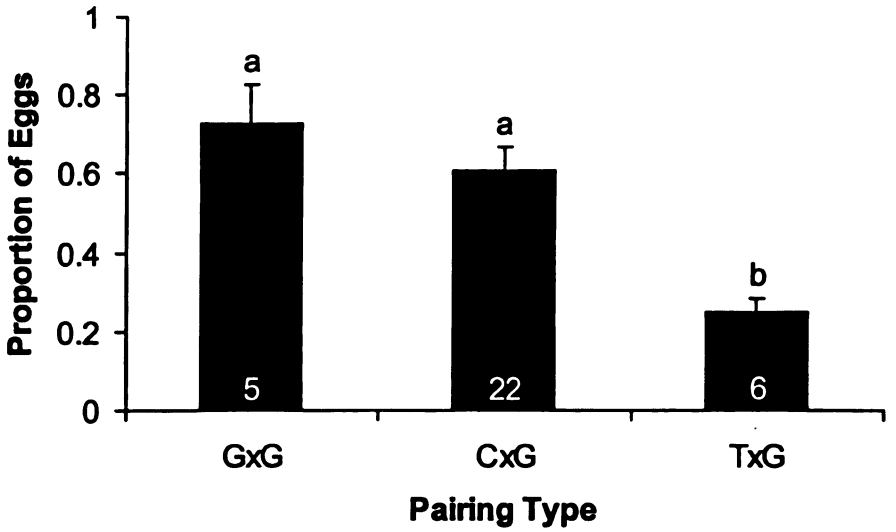


Figure 2.9. For all pairings producing at least one hatching larva, the mean proportion of viable eggs (hatching/total eggs). Values expressed are means, error bars are +1 s.e. and numbers in bars are numbers of pairings. Bars not sharing a letter are significantly different from each other at $p$=0.05. Female *glaucus* (G), *canadensis* (C), or *troilus* (T) were paired to wild-caught male *glaucus*, with the female listed first.

There were no differences between pairings involving hybrid *canadensis* × *glaucus* females and pairings involving females of either parental species (when paired to *canadensis* males) in the proportion lasting 30 minutes (Figure 2.10) or the proportion resulting in spermatophore deposition (Figure 2.11). There also were not differences between pairings involving hybrid *canadensis* × *glaucus* females and pairings involving females of either parental species in the proportion leading to oviposition (Figure 2.12) or the proportion leading to larvae (Figure 2.13). Pairings between hybrid females and wild *canadensis* males did not lead to a significantly lower egg hatchability than pairings with conspecific *canadensis* females, however both led to a lower hatchability than pairings with the heterospecific *glaucus* females (Figure 2.14).

The proportion of pairings leading to larvae, out of all pairings for which all of the first four measures were known (pairing duration, spermatophore deposition, whether

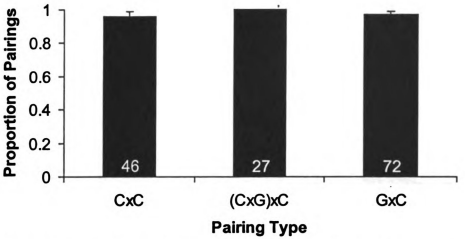


Figure 2.10. Out of all pairings that lasted a minimum of five minutes, the proportion of pairings lasting at least 30 minutes. Error bars are +1 s.d. and numbers in bars are sample sizes. Female *canadensis* (C), *glaucus* (G), or hybrid *canadensis* × *glaucus* (C × G) were paired to wild-caught male *canadensis*, with the female listed first.

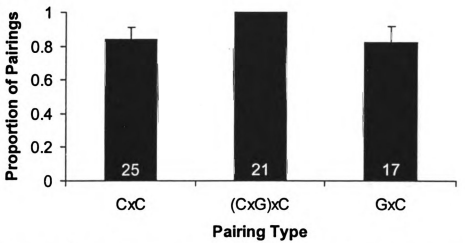


Figure 2.11. Out of all pairings lasting at least 30 minutes, the proportion of pairings resulting in spermatophore deposition. Error bars are +1 s.d. and numbers in bars are sample sizes. Female *canadensis* (C), *glaucus* (G), or hybrid *canadensis* × *glaucus* (C × G) were paired to wild-caught male *canadensis*, with the female listed first.

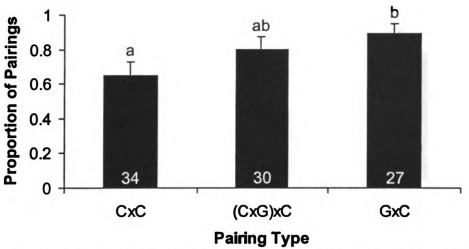


Figure 2.12. Out of all pairings with spermatophore deposition, the proportion of pairings leading to oviposition. Error bars are +1 s.d. and numbers in bars are sample sizes. Bars not sharing a letter are significantly different from each other at $p$=0.05. Female *canadensis* (C), *glaucus* (G), or hybrid *canadensis* × *glaucus* (C × G) were paired to wild-caught male *canadensis*, with the female listed first.

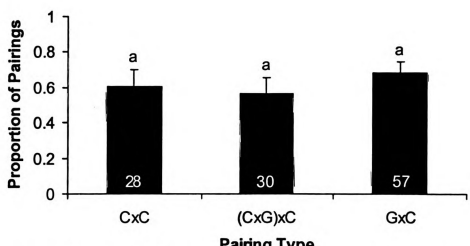


Figure 2.13. Out of all pairings leading to oviposition, the proportion of pairings leading to production of some larvae. Error bars are +1 s.d. and numbers in bars are sample sizes. Bars not sharing a letter are significantly different from each other at $p$=0.05. Female *canadensis* (C), *glaucus* (G), or hybrid *canadensis* × *glaucus* (C × G) were paired to wild-caught male *canadensis*, with the female listed first.

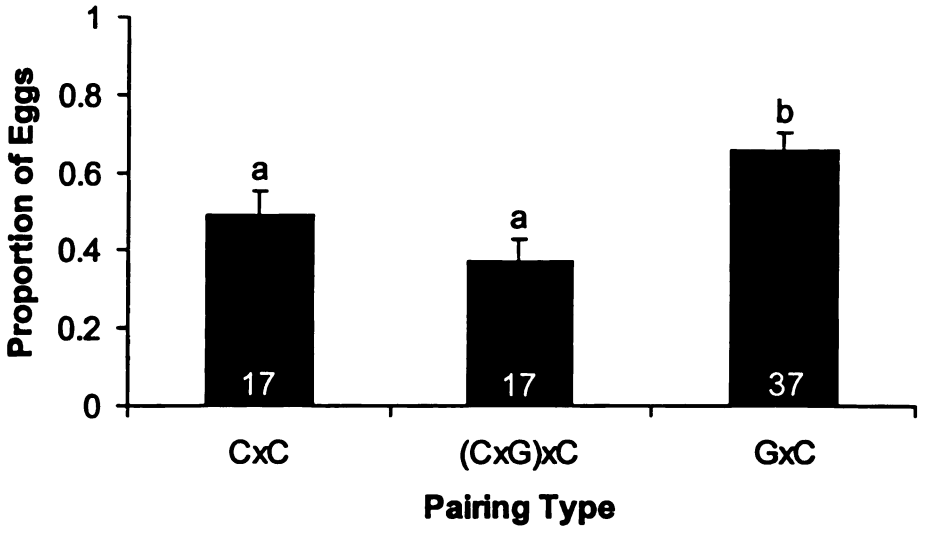


**Figure 2.14. For all pairings producing at least one hatching larva, the mean proportion of viable eggs (hatching/total eggs). Values expressed are means, error bars are +1 s.e. and numbers in bars are numbers of pairings. Bars not sharing a letter are significantly different from each other at $p$=0.05. Female *canadensis* (C), *glaucus* (G), or hybrid *canadensis* × *glaucus* (C × G) were paired to wild-caught male *canadensis*, with the female listed first.**

eggs had been laid, and whether larvae had been produced; Table 2.8) were multiplied by the average egg hatchabilities of each type of pairing (calculated in Table 2.9), to give a combined index of mating success (calculated in Table 2.10). Heterospecific pairings have the two highest index values, and conspecific pairings have two of the three lowest index values, so heterospecific pairings are not at a disadvantage with respect to this combined index of mating success (Table 2.10). One of the surprisingly low values of this index is for *glaucus* × *glaucus* wild pairings, with an index value of 0.08, the lowest value for any pairing type involving wild-caught males (Table 2.10). This low value is mainly due to the low frequency of pairings leading to larvae, out of those with oviposition (Table 2.8), which could have been affected by the low sample number for this pairing type.

Table 2.8. Types and numbers of pairs, each with a complete suite of data[1], and their reproductive success.

| Pairing Type (♀× ♂rigin) | Number of pairings, all lasting at least 5 minutes | Of those, the number lasting at least 30 minutes | Of those, the number with spermatophore deposition | Of those, the number with oviposition | Of those, the number with hatching larvae | Proportion of original pairings with hatching larvae |
|---|---|---|---|---|---|---|
| *canadensis* × *canadensis* wild (C × C wild) | 31 | 29 | 25 | 17 | 13 | 0.42 |
| *canadensis* × *canadensis* lab (C × C lab) | 44 | 41 | 36 | 27 | 5 | 0.11 |
| *glaucus* × *glaucus* wild (G × G wild) | 9 | 9 | 9 | 6 | 1 | 0.11 |
| *glaucus* × *glaucus* lab (G × G lab) | 25 | 24 | 23 | 20 | 10 | 0.40 |
| *canadensis* × *glaucus* wild (C × G wild) | 29 | 29 | 27 | 19 | 15 | 0.52 |
| *canadensis* × *glaucus* lab (C × G lab) | 21 | 21 | 19 | 13 | 7 | 0.33 |
| *glaucus* × *canadensis* wild (G × C wild) | 22 | 22 | 20 | 18 | 13 | 0.59 |
| *glaucus* × *canadensis* lab (G × C lab) | 16 | 15 | 12 | 9 | 3 | 0.19 |
| *troilus* × *glaucus* wild (T × G wild) | 9 | 9 | 8 | 7 | 6 | 0.67 |
| (*canadensis* × *glaucus*) × *canadensis* wild ((C × G) × C wild) | 21 | 21 | 21 | 16 | 11 | 0.52 |

[1]Additional pairings, lacking data from one or more of the above columns, are included in Figures 2.1 through 2.14 and in Tables 2.1 through 2.7.

Table 2.9. Egg hatchability of broods producing larvae.

| Pairing Type (♀× ♂rigin) | Number of eggs laid | Number of larvae hatching | Proportion of eggs hatching | Average hatchability for all broods |
|---|---|---|---|---|
| *canadensis* × *canadensis* wild | 23 | 8 | 0.35 | 0.49 ± 0.06, |
| (C × C wild) | 5 | 2 | 0.40 | N=17 |
| | 51 | 48 | 0.94 | |
| | 20 | 7 | 0.35 | |
| | 31 | 5 | 0.16 | |
| | 74 | 64 | 0.86 | |
| | 63 | 35 | 0.56 | |
| | 119 | 37 | 0.31 | |
| | 10 | 6 | 0.60 | |
| | 91 | 29 | 0.32 | |
| | 104 | 8 | 0.08 | |
| | 55 | 29 | 0.53 | |
| | 59 | 33 | 0.56 | |
| | 48 | 46 | 0.96 | |
| | 21 | 8 | 0.38 | |
| | 49 | 36 | 0.74 | |
| | 14 | 3 | 0.21 | |
| *canadensis* × *canadensis* lab | 37 | 3 | 0.08 | 0.40 ± 0.12, |
| (C × C lab) | 24 | 6 | 0.25 | N=7 |
| | 73 | 19 | 0.26 | |
| | 49 | 22 | 0.45 | |
| | 39 | 31 | 0.80 | |
| | 68 | 59 | 0.87 | |
| | 56 | 6 | 0.11 | |
| *glaucus* × *glaucus* wild | 34 | 17 | 0.50 | 0.72 ± 0.10, |
| (G × G wild) | 219 | 104 | 0.48 | N=5 |
| | 450 | 445 | 0.99 | |
| | 89 | 80 | 0.90 | |
| | 25 | 19 | 0.76 | |

Table 2.9 (cont'd).

| Pairing Type (♀× ♂origin) | Number of eggs laid | Number of larvae hatching | Proportion of eggs hatching | Average hatchability for all broods |
|---|---|---|---|---|
| *glaucus* × *glaucus* lab | 66 | 31 | 0.47 | 0.63 ± 0.07, |
| (G × G lab) | 75 | 65 | 0.87 | N=18 |
| | 72 | 1 | 0.01 | |
| | 107 | 13 | 0.12 | |
| | 17 | 5 | 0.29 | |
| | 389 | 261 | 0.67 | |
| | 188 | 161 | 0.86 | |
| | 85 | 66 | 0.78 | |
| | 15 | 4 | 0.27 | |
| | 27 | 22 | 0.82 | |
| | 84 | 63 | 0.75 | |
| | 126 | 121 | 0.96 | |
| | 9 | 7 | 0.78 | |
| | 63 | 61 | 0.97 | |
| | 70 | 63 | 0.90 | |
| | 54 | 22 | 0.41 | |
| | 68 | 36 | 0.53 | |
| | 45 | 40 | 0.89 | |

Table 2.9 (cont'd).

| Pairing Type (♀× ♂origin) | Number of eggs laid | Number of larvae hatching | Proportion of eggs hatching | Average hatchability for all broods |
|---|---|---|---|---|
| *canadensis* × *glaucus* wild (C × G wild) | 15 | 11 | 0.73 | 0.61 ± 0.06, N=22 |
| | 209 | 62 | 0.30 | |
| | 51 | 37 | 0.72 | |
| | 39 | 19 | 0.49 | |
| | 184 | 166 | 0.90 | |
| | 5 | 5 | 1.00 | |
| | 5 | 2 | 0.40 | |
| | 82 | 52 | 0.63 | |
| | 11 | 7 | 0.64 | |
| | 24 | 24 | 1.00 | |
| | 73 | 48 | 0.66 | |
| | 30 | 3 | 0.10 | |
| | 94 | 61 | 0.65 | |
| | 94 | 61 | 0.65 | |
| | 66 | 55 | 0.83 | |
| | 36 | 14 | 0.39 | |
| | 37 | 24 | 0.65 | |
| | 21 | 3 | 0.14 | |
| | 113 | 111 | 0.98 | |
| | 40 | 9 | 0.22 | |
| | 40 | 19 | 0.48 | |
| | 91 | 74 | 0.81 | |
| *canadensis* × *glaucus* lab (C × G lab) | 91 | 71 | 0.78 | 0.47 ± 0.10, N=10 |
| | 50 | 35 | 0.70 | |
| | 50 | 48 | 0.96 | |
| | 96 | 5 | 0.05 | |
| | 28 | 1 | 0.04 | |
| | 36 | 9 | 0.25 | |
| | 14 | 5 | 0.36 | |
| | 62 | 22 | 0.36 | |
| | 26 | 22 | 0.85 | |
| | 32 | 13 | 0.41 | |

Table 2.9 (cont'd).

| Pairing Type (♀× ♂origin) | Number of eggs laid | Number of larvae hatching | Proportion of eggs hatching | Average hatchability for all broods |
|---|---|---|---|---|
| *glaucus* × *canadensis* wild | 42 | 7 | 0.17 | 0.66 ± 0.04, |
| (G × C wild) | 205 | 196 | 0.96 | N=37 |
| | 398 | 293 | 0.74 | |
| | 146 | 102 | 0.70 | |
| | 115 | 86 | 0.75 | |
| | 83 | 37 | 0.45 | |
| | 148 | 128 | 0.86 | |
| | 102 | 42 | 0.41 | |
| | 132 | 16 | 0.12 | |
| | 39 | 36 | 0.92 | |
| | 95 | 41 | 0.43 | |
| | 125 | 79 | 0.63 | |
| | 129 | 126 | 0.98 | |
| | 70 | 34 | 0.49 | |
| | 23 | 5 | 0.22 | |
| | 47 | 37 | 0.79 | |
| | 113 | 94 | 0.83 | |
| | 86 | 79 | 0.92 | |
| | 97 | 94 | 0.97 | |
| | 99 | 52 | 0.52 | |
| | 89 | 26 | 0.29 | |
| | 97 | 95 | 0.98 | |
| | 350 | 225 | 0.64 | |
| | 203 | 184 | 0.91 | |
| | 82 | 22 | 0.27 | |
| | 29 | 19 | 0.66 | |
| | 102 | 102 | 1.00 | |
| | 56 | 6 | 0.11 | |
| | 170 | 139 | 0.82 | |
| | 50 | 44 | 0.88 | |
| | 163 | 126 | 0.77 | |
| | 67 | 54 | 0.81 | |
| | 27 | 5 | 0.18 | |
| | 124 | 74 | 0.60 | |
| | 209 | 157 | 0.75 | |
| | 271 | 230 | 0.85 | |
| | 254 | 224 | 0.88 | |

Table 2.9 (cont'd).

| Pairing Type (♀× ♂origin) | Number of eggs laid | Number of larvae hatching | Proportion of eggs hatching | Average hatchability for all broods |
|---|---|---|---|---|
| *glaucus* × *canadensis* lab | 185 | 66 | 0.36 | 0.28 ± 0.09, |
| (G × C lab) | 17 | 8 | 0.47 | N=4 |
| | 40 | 1 | 0.02 | |
| | 29 | 8 | 0.28 | |
| *troilus* × *glaucus* wild | 54 | 13 | 0.24 | 0.25 ± 0.03, |
| (T × G wild) | 98 | 38 | 0.39 | N=6 |
| | 30 | 6 | 0.20 | |
| | 96 | 27 | 0.28 | |
| | 40 | 10 | 0.25 | |
| | 135 | 18 | 0.13 | |
| (*canadensis* × *glaucus*) | 100 | 29 | 0.29 | 0.37 ± 0.06, |
| × *canadensis* wild | 30 | 9 | 0.30 | N=17 |
| ((C × G) × C wild) | 9 | 9 | 1.00 | |
| | 83 | 9 | 0.11 | |
| | 111 | 86 | 0.78 | |
| | 32 | 15 | 0.47 | |
| | 29 | 9 | 0.31 | |
| | 109 | 54 | 0.50 | |
| | 81 | 10 | 0.12 | |
| | 120 | 58 | 0.48 | |
| | 99 | 43 | 0.43 | |
| | 114 | 31 | 0.27 | |
| | 57 | 12 | 0.21 | |
| | 185 | 58 | 0.31 | |
| | 38 | 1 | 0.03 | |
| | 132 | 63 | 0.48 | |
| | 86 | 21 | 0.24 | |

Table 2.10. Calculation of combined index of mating success. For each pairing type, the proportion of pairings producing hatching larvae (Table 2.8) is multiplied by the average hatchability of broods producing larvae (Table 2.9).

| Pairing Type (♀× ♂rigin) | Out of pairings lasting at least 5 minutes, the proportion producing larvae | Average egg hatchability of pairings producing larvae | Combined index of mating success |
|---|---|---|---|
| C × C wild | 0.42 | 0.49 | 0.20 |
| C × C lab | 0.11 | 0.40 | 0.04 |
| G × G wild | 0.11 | 0.72 | 0.08 |
| G × G lab | 0.40 | 0.63 | 0.25 |
| C × G wild | 0.52 | 0.61 | 0.32 |
| C × G lab | 0.33 | 0.47 | 0.16 |
| G × C wild | 0.59 | 0.66 | 0.39 |
| G × C lab | 0.19 | 0.28 | 0.05 |
| T × G wild | 0.67 | 0.25 | 0.17 |
| (C × G) × C wild | 0.52 | 0.37 | 0.19 |

**Discussion**

I observed no reduction in pairing success (as indicated by copulation duration, spermatophore deposition, oviposition, and egg hatchability) for heterospecific pairings between *canadensis* and *glaucus*, indicating there is no postpairing, prezygotic reproductive isolation between these species when females have mated once. Pairings between *troilus* females and *glaucus* males were only less successful in average egg hatchability. The ability of species as phylogenetically separate as *troilus* and *glaucus* to pair successfully, and to do so with fairly high frequency, is quite impressive. These two species are probably behaviorally isolated in the wild (but see Deering & Scriber 1998; documents observation of a courtship and copulation between a tethered *canadensis* female and wild male of *Papilio palamedes*, a member of the *P. troilus* species group), but once behavior is superseded, even considerable physiological differentiation does not

prevent successful mating. The reduced egg hatchability that was observed (Figure 2.9) could be due either to an inability to fertilize many eggs (prezygotic isolation) or low egg viability (postzygotic isolation). Pairings between hybrid females and *canadensis* males were also very successful, with only slight reduction in egg hatchability, indicating very little reduction in fertility in hybrids (at least of this type).

These results do not address courtship or mate recognition early in a pairing, before the two have locked together. Reproductive isolation at this point would be best addressed under more natural conditions to minimize the effects of human handling. However, once pairs have locked together, they seem to progress well despite the artificial environment of the laboratory.

This study found that for larval production, the minimum copulation duration is 30 minutes, which matched the result of a previous study (Lederhouse et al. 1990). However, it is possible that the minimum duration could be shorter in nature. The pairings for this study were carried out at room temperature (75-85 F°), but in the wild, *Papilio* butterflies are usually found mating in the early afternoon, during the hottest part of the day. Warmer conditions might speed up the physiological processes of copulation, shortening the time required to mate successfully.

Lab-reared males have previously been found to be less reproductively successful than wild-caught males (Lederhouse et al. 1990). It was concluded that adult nutrition was to blame, and it was recommended that lab-reared males be fed honey water supplemented with amino acids and salts to provide the nutrients that males in the wild presumably obtain by puddling. However, in this study lab males were fed this solution, but they were still less successful than wild males in egg hatchability (Table 2.6, Table

2.7). Either the honey solution is still missing some important nutrient, or males in the wild benefit from some other factor that lab males do not get. This effect is also not due to inbreeding because most lab-reared individuals used in our lab are the offspring of wild-caught females.

Many females laid only unfertilized eggs, and even females that laid some fertilized eggs also laid many that were not fertile. This wastefulness of eggs is surprising, but in line with previous findings with *Papilio*, both for hand-paired butterflies (Clarke & Sheppard 1956) and for wild-caught females (Lederhouse & Scriber 1987). This suggests that females are dependent on males to provide adequate spermatophores and sperm in pairings, and that great variation in male (or male ejaculate) quality exists (Drummond 1984). However, they seem to have little ability to measure male quality (at least after a copulation has progressed) because females will lay eggs even with no spermatophore present or after short pairings. This seems surprising, but it is wise to avoid what Eberhard (1996) calls "fertilization myopia", the thinking that in the wild all copulations will lead to offspring and all eggs that females lay will be fertile. This will rarely be the case, so lab findings of 'wasted eggs' should not necessarily be shrugged off as the result of lab conditions.

Since females of both *canadensis* and *glaucus* often mate more than once in the wild (Lederhouse and Scriber 1989; Lederhouse 1995), another aspect of mating that could be very important is sperm competition (Birkhead & Møller 1998). Some insect females that have mated to two males, one conspecific and one heterospecific, will produce only conspecific offspring (Howard 1999). This potent reproductive barrier will be investigated in the following chapter.

In conclusion, heterospecific copulations between *canadensis* and *glaucus* were not less successful than conspecific, so there does not appear to be a postcopulatory, prezygotic barrier to gene flow between these species in singly-mated females.

# CHAPTER 3:

# DOES CONSPECIFIC SPERM HAVE PRECEDENCE IN *PAPILIO CANADENSIS* OR *P. GLAUCUS*?

## Introduction

There are postcopulatory, prezygotic barriers to gene flow that do not appear in singly-mated females. Groups of species of insects have been found where heterospecific pairings are no less successful than conspecific pairings when females mate only once, but when a female is paired to both a heterospecific male and a conspecific male, she produces only conspecific offspring, regardless of the order of the pairings (Howard 1999). This is called conspecific sperm precedence, and it can be a potent barrier to gene flow provided females can be expected to mate with multiple males (Howard et al. 1998).

The multiple-mating swallowtail butterfly species *Papilio glaucus* and *P. canadensis* interbreed to form viable, fertile hybrids (Lederhouse et al. 1989, Scriber et al. 1995). In the lab, heterospecific pairings are no less successful than conspecific pairings (Chapter 2). These two species can also form viable hybrids with the more distantly related western *Papilio* species *P. rutulus*, *P. eurymedon*, and *P. multicaudatus* (Scriber et al. 1995). Weak postzygotic barriers to gene flow between these species may indicate that prezygotic barriers isolate them (Sperling 1990).

The female reproductive system of the ditrysian Lepidoptera (Figure 1.2) might facilitate conspecific sperm precedence. Because males do not place sperm directly into the spermatheca of the female, they cannot directly displace the sperm of previous males

(Drummond 1984). Females may also be able to choose what sperm is sent to the spermatheca (Eberhard 1996).

Sperm precedence for an individual doubly-mated female can be expressed as $P_2$, the proportion of offspring produced after a second mating that was sired by the second male (Gwynne 1984). When there is first-male sperm precedence, $P_2$ will be close or equal to zero for most double pairings, and if last-male sperm precedence is the rule, $P_2$ will usually be close or equal to one. However, with conspecific sperm precedence, $P_2$ will be high when the last male was conspecific and low when the last male was heterospecific.

To look for conspecific sperm precedence in *glaucus* and *canadensis*, I paired virgin females twice, once to a conspecific male and once to a heterospecific male, and determined the paternity of offspring using allozyme electrophoresis. I also paired wild-caught females (that had presumably already mated in the wild to conspecific males) to heterospecific males. In addition to females and males of *canadensis* and *glaucus*, we also used males of the more distantly related species *rutulus*, *eurymedon*, and *multicaudatus*.

**Methods**

Both wild-caught and lab-reared male and female butterflies were used for pairings. Females and males of *glaucus* and *canadensis* were used, and males of *rutulus*, *eurymedon*, and *multicaudatus* were used. Through adulthood, female butterflies were fed a 20% honey solution and males were fed a 20% honey solution supplemented with amino acids and salts to increase fertility following Lederhouse et al. (1990). Lab-reared

males were not paired for at least two days following adult eclosion to allow reproductive maturation.

Lab-reared females were hand-paired to males, allowed to oviposit in plastic oviposition arenas lined with hostplant foliage (Scriber 1993), and remated after two to six days, again by hand-pairing, to a male of a different species. Females were then allowed to oviposit again. Table 3.1 shows the number and types of double-pairs made. Additionally, wild-caught females were allowed to oviposit, then remated after one to five days by hand-pairing to a male of a different species, and allowed to oviposit again. Table 3.2 shows the number and types of wild female rematings. Only females that were actively laying eggs were remated, and the duration of lab pairings were recorded.

Larvae from eggs laid both before and after rematings were collected and reared on black cherry (*Prunus serotina*) foliage, a common favorite of tiger swallowtail species. After reaching approximately the third instar, larvae were frozen at -80°C. Mothers and male mates were also stored frozen after death. Females were later dissected to determine how many spermatophores were present at death.

Lab and wild females producing larvae before remating were compared to those not producing larvae before remating with respect to success in laying eggs and producing larvae following remating. Data were analyzed using a contingency table analysis (PROC CATMOD; SAS Institute Inc. 1990).

Allozyme electrophoresis, following Hagen and Scriber (1991), was carried out on thin-layer cellulose acetate plates (Titan III, Helena Laboratories, Beaumont TX). Small larvae were homogenized whole in buffer, and the head and thorax of larger larvae were homogenized in buffer. With adult males, the distal half of the abdomen was used,

and with adult females, the proximal half of the abdomen was used (to avoid including male allozymes from spermatophores). The enzyme 6-phosphogluconate dehydrogenase (PGD) was stained for to determine paternity because there are diagnostic differences between the species of the *P. glaucus* species group in PGD allozymes (Hagen and Scriber 1991). There are other enzyme loci with diagnostic differences between species as well. Lactate dehydrogenase (LDH) and hexokinase (HK) can also be used to differentiate *glaucus* and *canadensis*, but LDH staining was faint for larvae and HK staining was uninterpretable for larvae. Staining of PGD was fainter for larvae than for adults, but it was clear and interpretable.

I verified the inheritance of *Pgd* as well as its expression in larvae. For the sixteen broods shown in Table 3.3, PGD allozymes were determined for the female, the first male to mate, and five to ten larvae produced before the female was remated. Expected offspring allozymes and proportions were compared to the actual offspring allozymes and numbers.

The paternity of offspring produced after remating was established by determining PGD allozymes of larvae produced after remating, several larvae produced before remating, and both of the males mated (in several cases the males were lost and not able to be checked). Sperm precedence for each brood was expressed as $P_2$, the proportion of larvae produced after the remating that were sired by the male used for remating. For several very large broods, I only determined the paternity of about twenty larvae produced after the remating rather than the entire brood. Broods where a female produced no hatching larvae before remating but did produce larvae after are not included in the tables of results, although the paternity of those larvae was determined.

When a doubly-mated female produces a brood of mixed paternity ($0<P_2<1$), the pattern of sperm use is of interest because it may be a clue for the mechanics of sperm replacement. For broods of mixed paternity where the production of larvae was spread out over more than one day, the numbers of larvae sired by each male for each day following remating was compared.

**Results**

The number of lab-reared females for each type of double-pairing is shown in Table 3.1. Out of 82 females, only 32 produced larvae both before and after being remated. The other 50 either laid no eggs after being remated, laid no hatching eggs after remating, or had laid no hatching eggs before being remated. Of the 32 females producing larvae both before and after being remated, five had been mated to males that shared allozymes (some interspecific introgression is found at the *Pgd* locus; Hagen et al. 1991, Chapter 5), making determining paternity of offspring impossible, leaving 27 broods where $P_2$ was determined.

The number of remated wild females is shown in Table 3.2. Out of 27 females, 20 produced larvae both before and after being remated. Six females laid no eggs after being remated. Almost all females that laid eggs after remating had larvae hatching from those eggs. All of the remated wild females produced larvae before being remated. Only one female that had produced larvae after being remated had undeterminable $P_2$, leaving 19 broods where $P_2$ was determined.

The comparison of allozymes of parents (a once mated female and her male mate) to larval offspring allozymes found that larvae had allozymes corresponding to their

Table 3.1. Types and numbers of double-paired lab-reared females and reproductive success following remating.

| Double-pairing type (♀× ♂1 × ♂2) | Number of double-paired females | Females laying eggs after remating | Females producing larvae after remating | Females producing larvae before and after remating | Females producing larvae before and after remating where $P_2$ could be determined |
|---|---|---|---|---|---|
| *canadensis* × *canadensis* × *glaucus* (C × C × G) | 18 | 17 | 8 | 6 | 5 |
| *canadensis* × *glaucus* × *canadensis* (C × G × C) | 13 | 12 | 6 | 5 | 5 |
| *glaucus* × *glaucus* × *canadensis* (G × G × C) | 17 | 13 | 9 | 6 | 6 |
| *glaucus* × *canadensis* × *glaucus* (G × C × G) | 20 | 18 | 13 | 8 | 5 |
| *canadensis* × *canadensis* × *eurymedon* (C × C × E) | 7 | 5 | 2 | 2 | 2 |
| *canadensis* × *eurymedon* × *canadensis* (C × E × C) | 4 | 4 | 2 | 2 | 2 |
| *glaucus* × *glaucus* × *multicaudatus* (G × G × M) | 1 | 1 | 1 | 1 | 1 |
| *glaucus* × *multicaudatus* × *glaucus* (G × M × G) | 1 | 1 | 1 | 1 | 0 |
| *glaucus* × *rutulus* × *glaucus* (G × R × G) | 1 | 1 | 1 | 1 | 1 |
| Total | 82 | 72 | 43 | 32 | 27 |

Table 3.2. Types and numbers of remated wild-caught females and reproductive success following remating.

| Remating type (♀ wild × ♂) | Number of remated wild-caught females | Females laying eggs after remating | Females producing larvae after remating | Females producing larvae before and after remating | Females producing larvae before and after remating where $P_2$ could be determined |
|---|---|---|---|---|---|
| *canadensis* wild × *glaucus* (C wild × G) | 8 | 7 | 6 | 6 | 6 |
| *glaucus* wild × *canadensis* (G wild × C) | 2 | 2 | 2 | 2 | 2 |
| *glaucus* wild × *eurymedon* (G wild × E) | 7 | 6 | 6 | 6 | 5 |
| *glaucus* wild × *multicaudatus* (G wild × M) | 4 | 3 | 3 | 3 | 3 |
| *glaucus* wild × *rutulus* (G wild × R) | 6 | 3 | 3 | 3 | 3 |
| Total | 27 | 21 | 20 | 20 | 19 |

parents (Table 3.3). The sample numbers are not high enough to be able to expect to see the actual frequencies in a large population, but based on these broods there is no reason to suspect non-Mendelian inheritance.

There was no significant difference in likeliness to lay eggs after being remated between lab females laying hatching eggs, lab females laying non-hatching eggs, and wild females laying hatching eggs before being remated (Figure 3.1). However, of females laying eggs after remating, females that had laid fertile eggs before the remating were significantly more likely to lay fertile eggs after the remating than females that had laid no hatching eggs before being remated. Wild-caught females were slightly more likely to lay hatching eggs after being remated than lab females that had been laying hatching eggs ($p$=0.0918).

Most of the broods that $P_2$ was determined for had $P_2$=0 (34 of 46 broods) (Table 3.4, Table 3.5). These broods were spread out through the different pairing types and female origins. However, there were cases of $P_2$>0 through most of the double pairing types. There were seven broods of mixed paternity.

Durations of second pairings were recorded. No second pairing lasting for fewer than 30 minutes resulted in sperm replacement (Table 3.4, Table 3.5). No female found to be carrying only one spermatophore showed any sperm replacement either. However, most second pairings lasted for longer than 30 minutes, and most females were found carrying two (or more for wild caught females) spermatophores, and even in many of these cases $P_2$ was equal to zero.

Table 3.3. Transmission and expression of PGD in larval offspring. PGD is X-linked in *Papilio*, where females are XY and males are XX. Homozygotes of PGD cannot be distinguished from hemizygotes in larvae because their sex is unknown.

| Pairing type (♀× ♂) | Pairing number | Female PGD allozymes | Male PGD allozymes | Expected offspring PGD allozymes and proportions | Actual offspring PGD allozymes and numbers |
|---|---|---|---|---|---|
| C × C | 14100 | -125 | -125/-125 | 1(-125) | 6(-125) |
| | 14251 | -125 | -125/-125 | 1(-125) | 7(-125) |
| | 14252 | -125 | -125/-80 | 0.25(-125/-80) : 0.5(-125) : 0.25(-80) | 3(-125/-80) : 2(-125) : 2(-80) |
| G × G | 14288 | -100 | -100/-100 | 1(-100) | 7(-100) |
| | 14289 | -100 | -100/-100 | 1(-100) | 7(-100) |
| | 14321 | -100 | -100/-100 | 1(-100) | 7(-100) |
| C × G | 13077 | -125 | -100/-100 | 0.5(-125/-100) : 0.5(-100) | 3(-125/-100) : 2(-100) |
| | 14279 | -125 | -100/-100 | 0.5(-125/-100) : 0.5(-100) | 5(-125/-100) : 1(-100) |
| | 14284 | -125 | -100/-100 | 0.5(-125/-100) : 0.5(-100) | 2(-125/-100) : 4(-100) |
| G × C | 14085 | -100 | -125/-125 | 0.5(-125/-100) : 0.5(-125) | 3(-125/-100) : 4(-125) |
| | 14086 | -100 | -125/-125 | 0.5(-125/-100) : 0.5(-125) | 2(-125/-100) : 4(-125) |
| | 14192 | -100 | -125/-125 | 0.5(-125/-100) : 0.5(-125) | 4(-125/-100) : 2(-125) |
| C × E | 14256 | -125 | -180/-150 | 0.25(-180/-125) : 0.25(-150/-125) : 0.25(-180) : 0.25(-150) | 0(-180/-125) : 2(-150/-125) : 3(-180) : 2(-150) |
| | 14259 | -125 | -150/-110 | 0.25(-150/-125) : 0.25(-125/-110) : 0.25( -150) : 0.25(-110) | 3(-150/-125) : 3(-125/-110) : 1( -150) : 3(-110) |

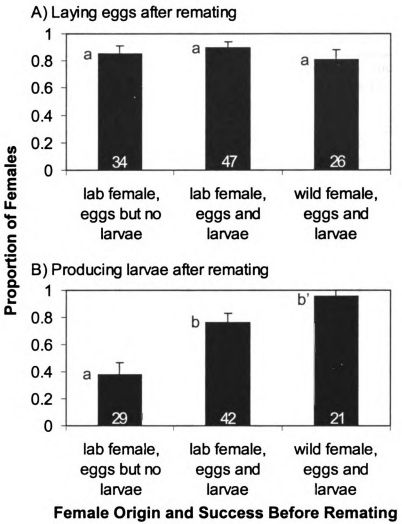


Figure 3.1. Reproductive success of multiply-mated females following remating asa a function of female origin and success before remating. A) Proportion of females laying eggs after remating. B) Of females laying eggs, the proportion producing larvae. Error bars are +1 s.d., numbers within bars are number of females, and bars with the same letter are not significantly different at the $p$=0.05 level. (Note: b is significantly different than b' at $p$=0.0918)

Table 3.4. Sperm precedence (the proportion of offspring that were sired by the second male; $P_2$) for double-paired lab-reared females. Also indicated are the number of larvae produced after remating that had paternity determined (N), origin of the male used for remating, the days between pairings, the duration of the second mating, and the number of spermatophores present in the female at death.

| Double-pairing type (♀× ♂1 × ♂2) | Female number | Days between pairings | Male origin | Duration of second mating (minutes) | Spermatophores present | $P_2$ | N |
|---|---|---|---|---|---|---|---|
| C × C × G | 13088 | 3 | lab | 65 | 2 | 1 | 15 |
| | 13093 | 3 | lab | 15 | 1 | 0 | 2 |
| | 13100 | 3 | lab | 100 | 2 | 0 | 27 |
| | 14100 | 2 | lab | 26 | 1 | 0 | 61 |
| | 14197 | 3 | lab | 60 | 2 | 0.2 | 5 |
| C × G × C | 13077 | 6 | lab | 108 | 2 | 0 | 1 |
| | 14278 | 3 | lab | 59 | 2 | 0 | 7 |
| | 14279 | 3 | lab | 35 | 1 | 0 | 22 |
| | 14284 | 2 | lab | >43 | 2 | 0 | 12 |
| | 14093 | 4 | wild | 57 | 2 | 1 | 21 |
| G × G × C | 14280 | 5 | lab | 93 | 1 | 0 | 16 |
| | 14281 | 2 | lab | >36 | 2 | 0 | 21 |
| | 14287 | 2 | lab | >38 | 2 | 0 | 23 |
| | 14288 | 3 | lab | 62 | 2 | 0.36 | 11 |
| | 14289 | 3 | lab | 63 | 2 | 0 | 26 |
| | 14321 | 3 | lab | 106 | 1 | 0 | 14 |
| G × C × G | 12328 | 3 | lab | (?) | 2 | 0.82 | 11 |
| | 14103 | 2 | lab | 65 | 2 | 0 | 1 |
| | 14192 | 4 | lab | 85 | 2 | 0 | 72 |
| | 14085 | 2 | wild | 87 | 2 | 0 | 26 |
| | 14086 | 4 | wild | >30 | 2 | 0 | 23 |
| C × C × E | 14251 | 4 | wild | >41 | 2 | 0 | 21 |
| | 14252 | 2 | wild | 73 | 2 | 0.93 | 14 |
| C × E × C | 14256 | 2 | wild | >85 | 1 | 0 | 21 |
| | 14259 | 2 | wild | >91 | 2 | 1 | 5 |
| G × G × M | 14277 | 4 | wild | >48 | 2 | 0 | 3 |
| G × R × G | 14381 | 1 | wild | 99 | 2 | 0 | 19 |

Table 3.5. Sperm precedence (the proportion of offspring that were sired by the second male; $P_2$) for remated wild-caught females. Also indicated are the number of larvae produced after remating that had paternity determined (N), origin of the male used for remating, the days between collection of the female and remating, the duration of the remating, and the number of spermatophores present in the female at death.

| Remating type (♀ wild × ♂) | Female number | Days until remating | Male origin | Duration of remating (minutes) | Spermatophores present | $P_2$ | N |
|---|---|---|---|---|---|---|---|
| C wild × G | 14000 | 1 | wild | >45 | 2 | 1 | 2 |
| | 14004 | 3 | wild | 115 | 3 | 0 | 1 |
| | 14005 | 3 | wild | 42 | 2 | 0 | 7 |
| | 14010 | 4 | wild | 33 | (?) | 0 | 14 |
| | 14017 | 3 | wild | 60 | 3 | 0.14 | 7 |
| | 14024 | 3 | wild | 72 | 3 | 0 | 5 |
| G wild × C | 14330 | 2 | lab | 42 | 1 | 0 | 20 |
| | 14331 | 2 | lab | 49 | 2 | 0 | 20 |
| G wild × E | 12483 | 1 | wild | >40 | 2 | 0 | 3 |
| | 12484 | 1 | wild | >40 | 4 | 0.07 | 14 |
| | 12485 | 1 | wild | 54 | 2 | 0.12 | 8 |
| | 14301 | 4 | wild | 64 | 1 | 0 | 22 |
| | 14294 | 4 | wild | >75 | 2 | 0 | 21 |
| G wild × M | 12487 | 5 | wild | >40 | 2 | 1 | 1 |
| | 12488 | 5 | wild | >40 | (?) | 0 | 2 |
| | 12496 | 5 | wild | >40 | 3 | 0 | 4 |
| G wild × R | 12490 | 5 | wild | 66 | 3 | 0 | 2 |
| | 12494 | 1 | wild | 27 | 1 | 0 | 11 |
| | 12590 | 2 | wild | (?) | 2 | 0 | 3 |

There were eleven females that produced larvae after remating, but had produced no larvae before remating. For three of those females, $P_2$ could not be determined. For seven of the remaining eight females, $P_2$ was equal to one. The eighth, female number 14133, was a *glaucus* female who had been paired first to a wild *canadensis* male, laid 37 infertile eggs, and was then paired to a lab *glaucus* male. After the remating, she produced a brood with $P_2$=0.27. This indicates that in most of the cases where a female laying no fertile eggs mates again and starts to produce fertile eggs, she will exclusively be using the sperm of the most recent male. However, in some cases she might be using sperm from the earlier male as well, even though before remating that sperm was not being successfully utilized.

Of the seven mixed broods, three could be divided up by the day that offspring were produced (Figure 3.2). All three of these had one larva produced the first day following remating that was sired by the first male, but two of the three had larvae produced on later days that had been sired by the first male as well. One brood, 14252, appears to follow the model of the first egg produced following remating being fertilized by the first male, followed by eggs fertilized by the second male.

**Discussion**

I found sperm replacement to be possible in remated *Papilio* females, but more commonly females continued to exclusively use sperm from the original mating. Heterospecific males were no less likely to replace sperm from a previous mating than conspecific males (and conversely, remated females were just as likely to continue to use heterospecific sperm from a first mating as conspecific sperm), meaning there was no

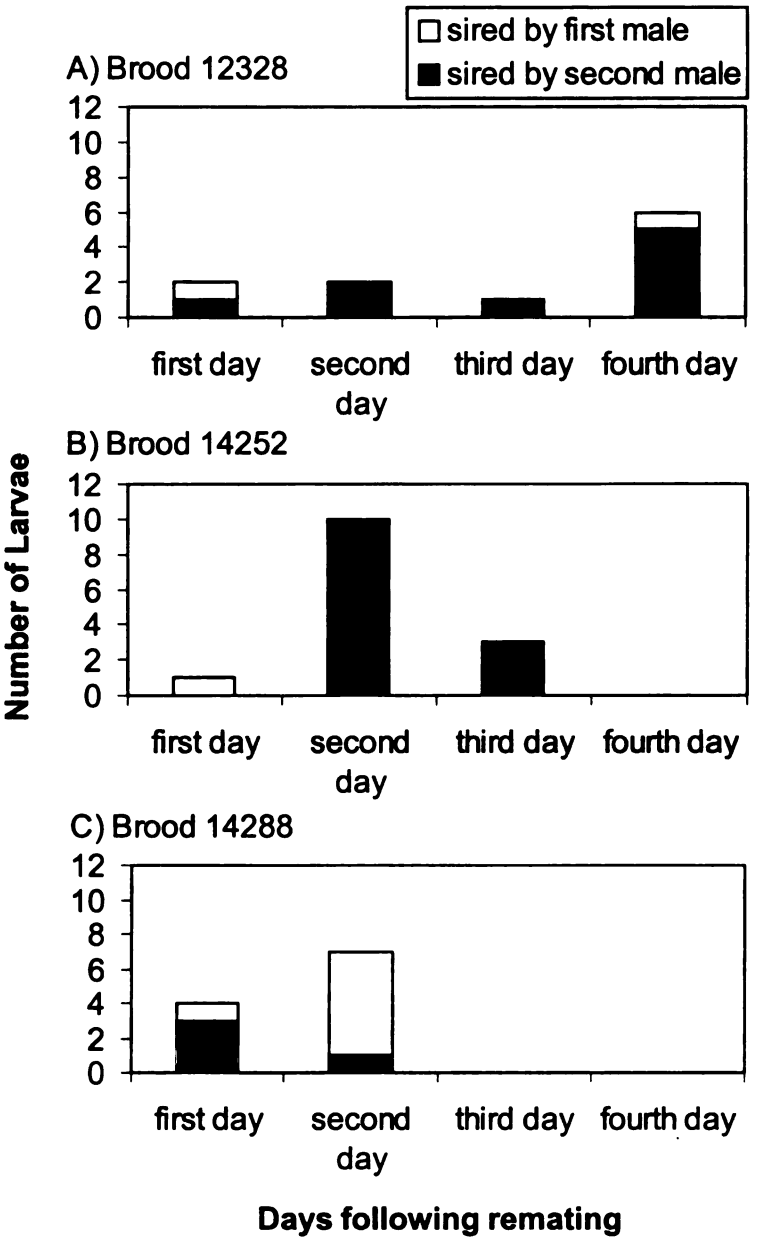


Figure 3.2. Number of larvae sired by each male and the days following remating of their production by females producing mixed broods: A) Brood 12328; B) Brood 14252; C) Brood 14288.

evidence for conspecific sperm precedence in *canadensis* or *glaucus*. The general pattern seems to be first-male sperm precedence, although with the variability in precedence found in many insects (Simmons & Siva-Jothy 1998). First-male priority is further supported by the fact that female reproductive success after remating was influenced by success before remating (Figure 3.1).

The failure of second matings to sire offspring is likely due to many of the same limitations on pairing success found in first pairings (Chapter 2). Other factors may enhance this as well. Large spermatophores may act as temporary mating plugs, as they seem to in some insects (Lorch et al. 1993), similar to the permanent mating plugs some *Papilio* males produce (Orr 1995). Large spermatophores might in the wild prolong the time until a female solicits another mating, as in bushcrickets (Wedell 1993). Thus increased time between pairings might increase the success of second matings. The use of lab males also may have reduced replacement success, although wild males were generally quite unsuccessful at replacing paternity as well.

In conclusion, I did not find that conspecific sperm has precedence in either *canadensis* or *glaucus*. Along with the results of Chapter 2, this means that there is no evidence for postpairing, prezygotic barriers to gene flow between these species.

# CHAPTER 4:

# HIGH LEVELS OF GENE FLOW BETWEEN POPULATIONS OF THE CANADIAN SWALLOWTAIL, *PAPILIO CANADENSIS*

## Introduction

Hybrid zones are clines maintained by a balance between gene flow and barriers to gene flow (Barton & Hewitt 1985). This means that in addition to studying reproductive isolation between the species involved, it is important to study dispersal and gene flow within both species as well as across the hybrid zone. Potential gene flow between species (if reproductive isolation, habitat differences, and any other barriers to interbreeding were to suddenly vanish) is equivalent to the actual gene flow within each of the species. If the potential for gene flow between species is high, then the barriers that isolate them must be quite strong.

The swallowtail butterflies *Papilio canadensis* and *P. glaucus* have ranges that meet at a narrow hybrid zone, and have overlapping flight times. There is no postpairing, prezygotic reproductive isolation (Chapters 2 & 3), and male behavior (*canadensis* males are more attracted to *glaucus* females than *canadensis* females, Deering 1998) might even increase gene flow between species. Hybrids are viable and fertile (Hagen & Scriber 1995), so as yet no strong barriers to gene flow have been found. However, if gene flow within both of the species is low, the differences between them could be maintained by weaker barriers (Barton & Hewitt 1985).

Evidence for high gene flow between widely-distributed *glaucus* populations has been found (Bossart & Scriber 1995), but it has not yet been investigated exclusively in

*canadensis* populations. Because of the biology of *canadensis*, there may be lower gene flow between its populations than between *glaucus* populations. Individuals of *canadensis* are typically smaller (possibly indicating lower resources for dispersal flights), undergo obligate pupal diapause (resulting in only one generation per year), and face a more time-limited growing period (Scriber 1994), all of which could reduce dispersal and gene flow in *canadensis* relative to *glaucus*.

One way to test the strength of gene flow is to sample populations separated by natural barriers. For example, in checkerspot butterflies gene flow is limited between populations in mountain areas, but not between plateau populations (Britten et al. 1995). The most significant natural barriers in the Great Lakes region are the lakes themselves. Lakes Michigan and Huron have been found to reduce gene flow between populations of the butterfly *Limenitis arthemis* (Waldbauer & Sternburg 1988), and may do so in other insect species as well.

A popular approach to studying gene flow has been to estimate it from geographic patterns of allele distribution (Slatkin 1987). A classical technique for this has been to use *F*-statistics (Wright 1931). Using some codominant, genetic characteristic, allele and genotype frequencies are determined from samples of individuals from several populations. If all individuals are treated as members of a single breeding population, the reduction in heterozygosity relative to Hardy-Weinberg equilibrium is calculated and expressed as $F_{IT}$. If there are fewer heterozygotes than expected, $F_{IT}$ will be positive, and if there are more than expected, it will be negative. $F_{IT}$ can be broken down into two components: $F_{IS}$, which is the reduction in heterozygotes within the subpopulations, and $F_{ST}$, which is the reduction in heterozygotes due to the population being divided into

subpopulations. If $F_{ST}$ is significantly larger than zero, it is an indication of reduced gene flow between subpopulations. Another indirect method to estimate gene flow is to statistically compare allele frequencies across populations (Raymond & Rousset 1995).

To estimate levels of gene flow between *canadensis* populations, I sampled populations throughout the Great Lakes region. Allozyme electrophoresis was used to determine allele and genotype frequencies at four enzyme loci, and *F*-statistics and other statistical methods were used to look for genetic structure (reduced gene flow) between populations.

**Methods**

Individuals were collected from six locations in the range of *canadensis* throughout the Great Lakes region: one from northeast Minnesota (Cook Co.), two from the Upper Peninsula of Michigan (Gogebic Co., Dickinson Co.), and three from the Lower Peninsula of Michigan (Charlevoix Co., Mason Co., Isabella Co.) (Figure 4.1). All specimens were collected between 14 May and 23 June of 1998 (peak flight time for *canadensis* in Michigan), and stored at -80°C.

Allozyme electrophoresis protocols followed Hagen and Scriber (1991). Samples were prepared by grinding the distal half of the abdomen for males or the proximal half of the abdomen for females (to avoid including spermatophore proteins from male mates) in 100μL buffer (0.1M tris, 1.07mM EDTA, 0.15mM NAD, 0.13mM NADP, 35.75mM 2-mercaptoethanol, pH 7.0) and centrifuging for 10 minutes at 16,000 × *g*. Allozymes were separated by electrophoresis on thin layer cellulose acetate plates (Titan III, Helena

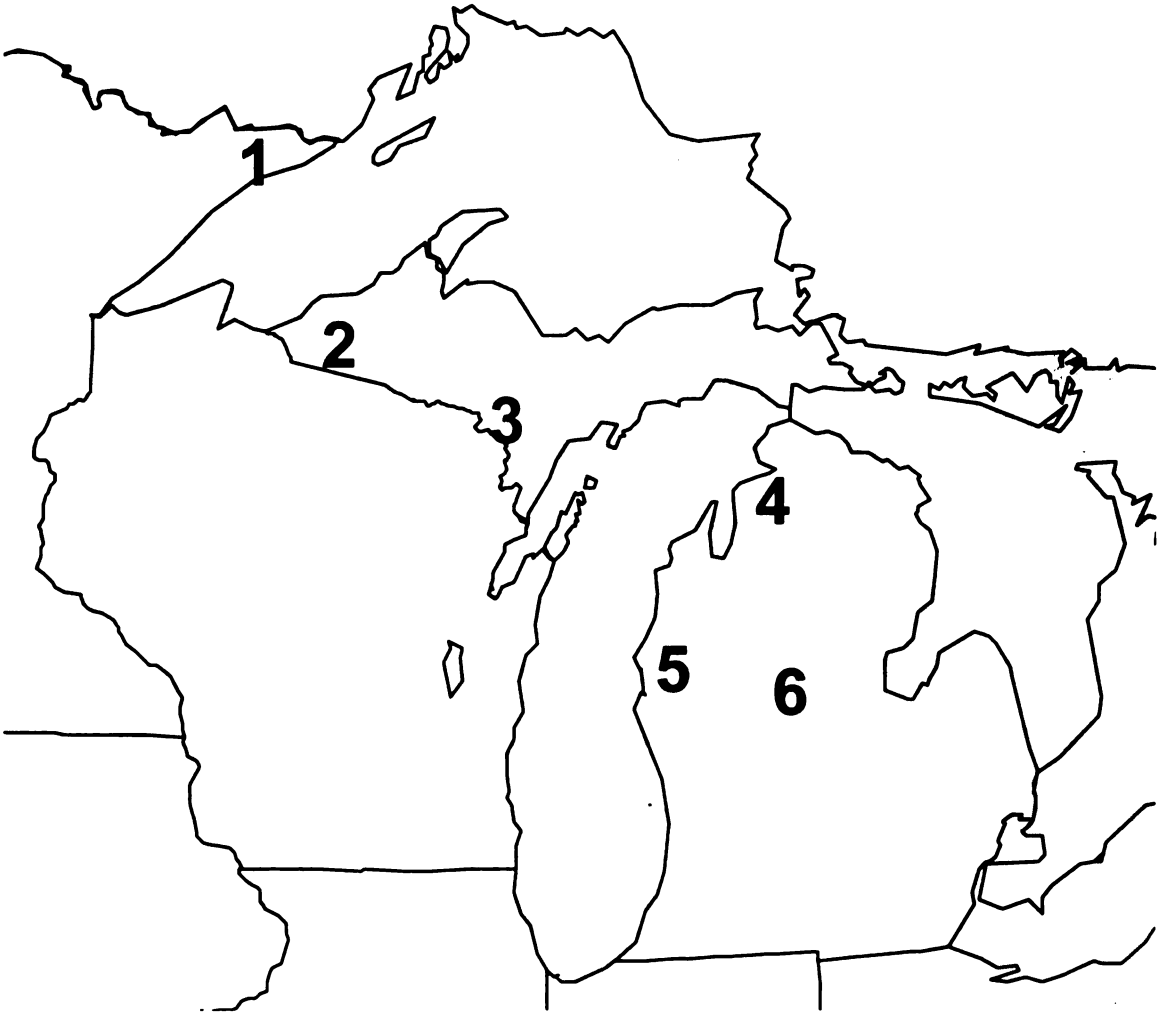


Figure 4.1. Sample sites. 1: Cook Co., Minnesota; 35 males. 2: Gogebic Co., Michigan; 36 males, 1 female. 3: Dickinson Co., Michigan; 48 males, 20 females. 4: Charlevoix Co., Michigan; 50 males, 18 females. 5: Mason Co., Michigan; 50 males, 15 females. 6: Isabella Co., Michigan; 50 males, 14 females.

Laboratories, Beaumont, TX). The four enzymes used (GPI, PGM, HBDH, and PGD) and the running conditions for each are shown in Table 4.1.

Enzyme stains followed Richardson et al. (1986). Gels were scored as in Hagen & Scriber (1991). The most common allozyme for each enzyme was given score '100', the origin (where samples had originally been applied) was given score '0', and all other allozymes were given a score corresponding to their location relative to these two points. Every sample plate was run with at least two previously scored samples to act as internal standards. These relative migration distance scores were then used as names for different alleles at the enzyme gene locus.

The program Genepop v3.1 (Raymond and Roussett 1995) was used to test for linkage disequilibrium, Hardy-Weinberg equilibrium, and allele frequency differences between locations. The program Fstat v2.8 (Goudet 1997) was used to calculate Wright's *F*-statistics and standard errors.

Table 4.1. Enzymes resolved and running conditions used.

| Enzyme | Name (E.C. Number) | Buffer* | Origin | Voltage | Time |
|---|---|---|---|---|---|
| GPI | Glucose phosphate isomerase (5.3.1.9) | I | cathode | 275V | 45 min. |
| PGM | Phosphoglucomutase (2.7.5.1) | I | cathode | 275V | 45 min. |
| HBDH | Hydroxybutyrate dehydrogenase (1.1.1.30) | D | anode or cathode** | 300V | 90 min. |
| PGD | 6-Phosphogluconate dehydrogenase (1.1.1.44) | D | anode or cathode** | 300V | 90 min. |

*Buffers (as in Richardson et al. 1986): I=25mM tris, 192mM glycine, pH 8.5; D=15mM tris, 5mM EDTA, 10mM $MgCl_2$, 5.5mM boric acid, pH 7.8.
**Under these conditions, HBDH and PGD migrated towards the center of the plate regardless of origin.

**Results**

When two genes are located close to each other on the same chromosome, they will tend to be inherited together, acting like a single gene. Within individuals, if certain alleles at one gene tend to be associated with certain alleles at other genes, they are probably being inherited as a single unit. This state is called linkage disequilibrium between those genes. If two genes indicate similar geographic patterns of allele distribution, they are only independent sources of information if there is not linkage disequilibrium between those genes. There were no significant *p*-values from chi-square tests of linkage disequilibrium between the four enzyme loci used here (Table 4.2), indicating that these four loci can be taken as independent sources of information.

Table 4.2. Chi-square values from tests for linkage of enzyme loci. The null hypothesis was $H_0$: genotypes at one locus are distributed independently from genotypes at the other locus.

| Locus pair | $\chi^2$ | df | *P*-value |
|---|---|---|---|
| *Gpi & Pgm* | 9.845 | 12 | 0.630 |
| *Gpi & Hbdh* | 10.307 | 12 | 0.589 |
| *Pgm & Hbdh* | 4.386 | 12 | 0.975 |
| *Gpi & Pgd* | 14.229 | 12 | 0.286 |
| *Pgm & Pgd* | 9.555 | 12 | 0.655 |
| *Hbdh & Pgd* | 17.025 | 12 | 0.149 |

Hardy-Weinberg equilibrium is a neutral state for polymorphic genes: one allele is not more likely to be selected against (either by natural or sexual selection) or enter or leave an area than other alleles. *P*-values for deviations from Hardy-Weinberg equilibrium are nonsignificant at $p$=0.05 in most locations for all loci (Table 4.3). However, there are significant deviations from equilibrium in populations for both PGM (in Cook Co., MN and Dickinson Co., MI) and HBDH (in Gogebic Co., MI and Isabella Co., MI), meaning that equilibrium cannot be assumed for all loci in all populations. However, in these cases, it is the frequency of genotypes involving rare alleles that deviates from Hardy-Weinberg equilibrium, meaning that these deviations might be affected by sample sizes. None of the loci deviate from equilibrium in all populations, and no population deviates from equilibrium at all loci. This means that forces acting on these loci (selection, assortative mating, etc.) that would confound gene flow measures are weak or nonexistent.

Table 4.3. *P*-values from tests of Hardy-Weinberg equilibrium. For each locus in each population, Genepop tests the null hypothesis of Hardy-Weinberg equilibrium.

| Location | GPI | PGM | HBDH | PGD |
|---|---|---|---|---|
| Cook Co., MN | 0.889 | 0.014 | 0.054 | 1 |
| Gogebic Co., MI | 1 | 0.429 | 0.035 | 1 |
| Dickinson Co., MI | 0.123 | 0.004 | 1 | 0.397 |
| Charlevoix Co., MI | 0.312 | 0.270 | 0.576 | 1 |
| Mason Co., MI | 0.742 | 0.845 | 0.598 | 0.076 |
| Isabella Co., MI | 0.492 | 0.777 | 0.006 | 1 |

Allozyme frequencies are similar in all six *canadensis* populations for all four loci (Figure 4.2, Figure 4.3, Figure 4.4, Figure 4.5). However, some frequency differences are seen (e.g. the frequency of $GPI^{100}$ is near 90% in Isabella Co., MI but less than 84% in the other locations, Figure 4.2), and there are significant differences between some populations at all four loci. Significant overall allele frequency differences are found for GPI, and marginally significant overall differences are found for PGD. Nevertheless, there is no general pattern of populations separated by the lakes being significantly different, and neighboring populations are as likely to be different as separated populations. Two populations with allele frequencies significantly different at one locus are generally not different at other loci.

Wright's *F*-statistics for these six populations are shown in Table 4.4. All $F_{ST}$-values are less than 0.01. $F_{ST}$ for PGM was calculated to be less than zero, and for the other three enzymes, $F_{ST}$ was within its standard error's range of zero. This indicates that there is little significant reduction in heterozygosity due to population subdivision. There may still be genetic structure in these populations (because $F_{ST}$ for three of the four loci is greater than zero), but if so, it is probably slight.

Table 4.4. Wright's *F*-statistics for six *canadensis* populations through the Great Lakes region. Standard errors were obtained by jackknifing over populations, and are indicated in parentheses.

| Locus | $F_{IS}$ (s.e.) | | $F_{ST}$ (s.e.) | | $F_{IT}$ (s.e.) | |
|---|---|---|---|---|---|---|
| GPI | 0.002 | (0.015) | 0.009 | (0.009) | 0.011 | (0.016) |
| PGM | 0.051 | (0.044) | -0.004 | (0.002) | 0.046 | (0.043) |
| HBDH | 0.108 | (0.076) | 0.002 | (0.008) | 0.110 | (0.078) |
| PGD | -0.016 | (0.039) | 0.005 | (0.007) | -0.011 | (0.039) |

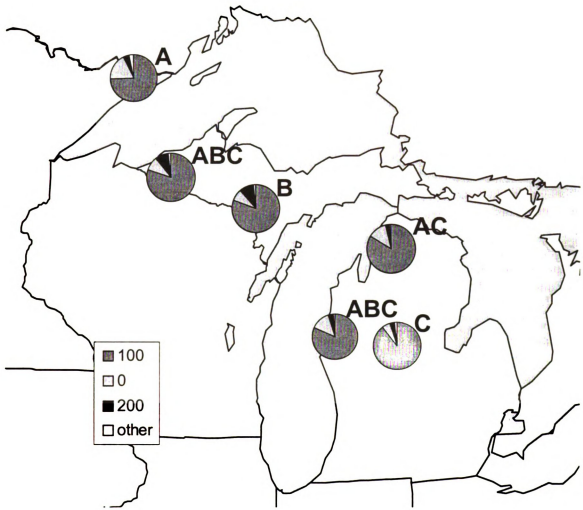


Figure 4.2. GPI allozyme frequencies for six sampled *canadensis* populations. Populations not sharing a letter are significantly different at $p$=0.05. The $P$-value for the test of overall allele differentiation is 0.004.

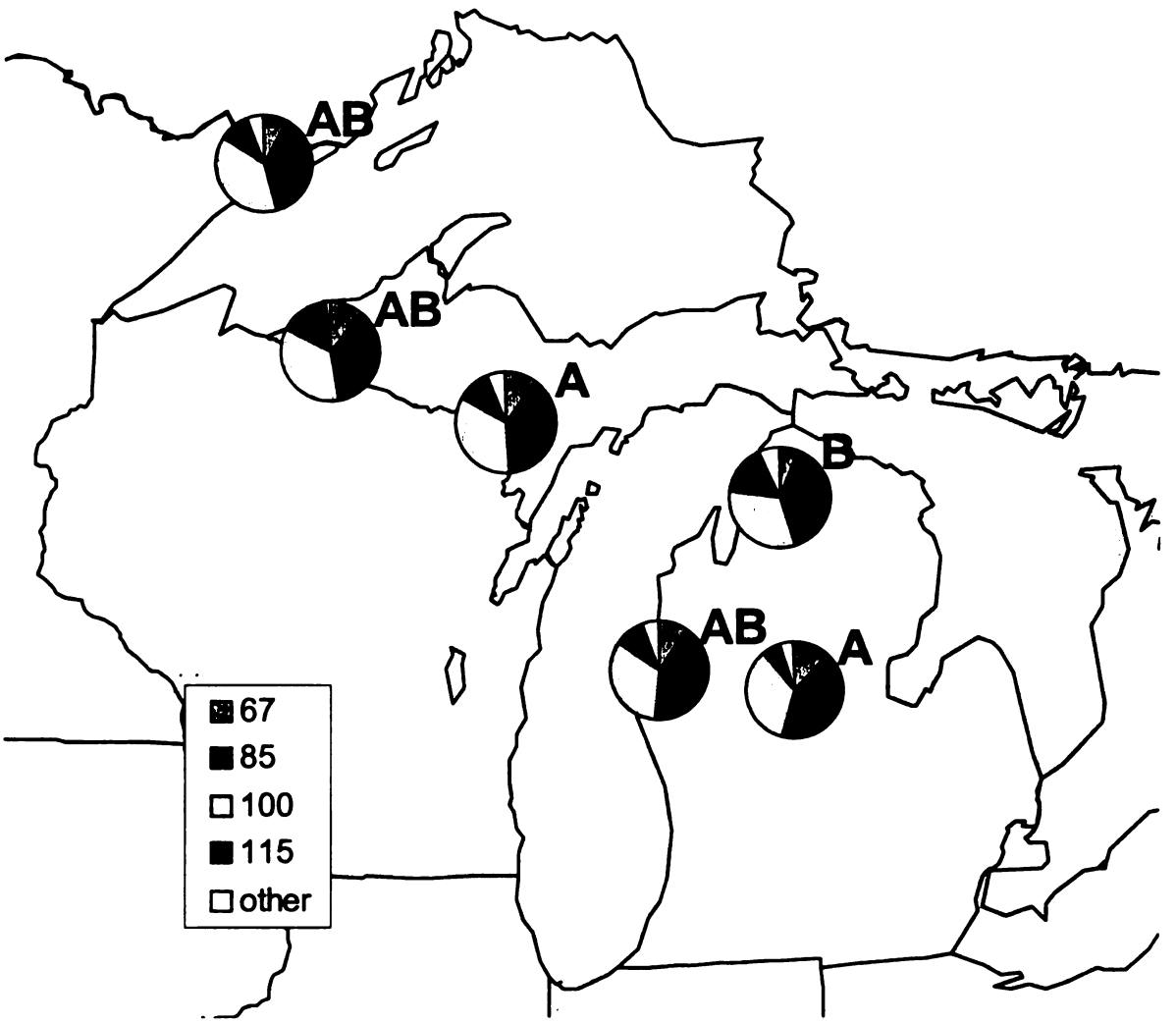


Figure 4.3. PGM allozyme frequencies for six sampled *canadensis* populations. Populations not sharing a letter are significantly different at $p$=0.05. The $P$-value for the test of overall allele differentiation is 0.561.

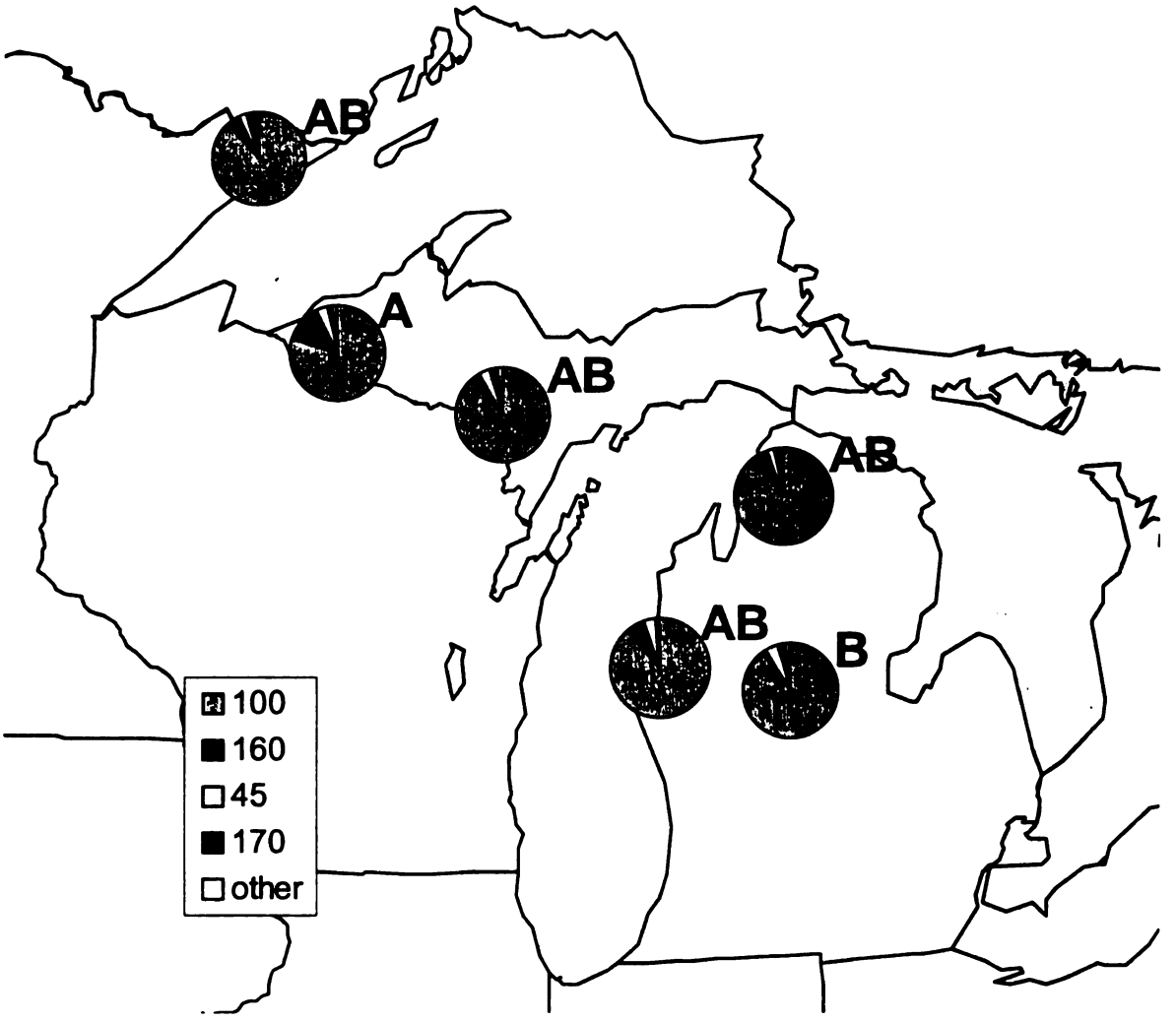


Figure 4.4. HBDH allozyme frequencies for six sampled *canadensis* populations. Populations not sharing a letter are significantly different at $p$=0.05. The $P$-value for the test of overall allele differentiation is 0.185.

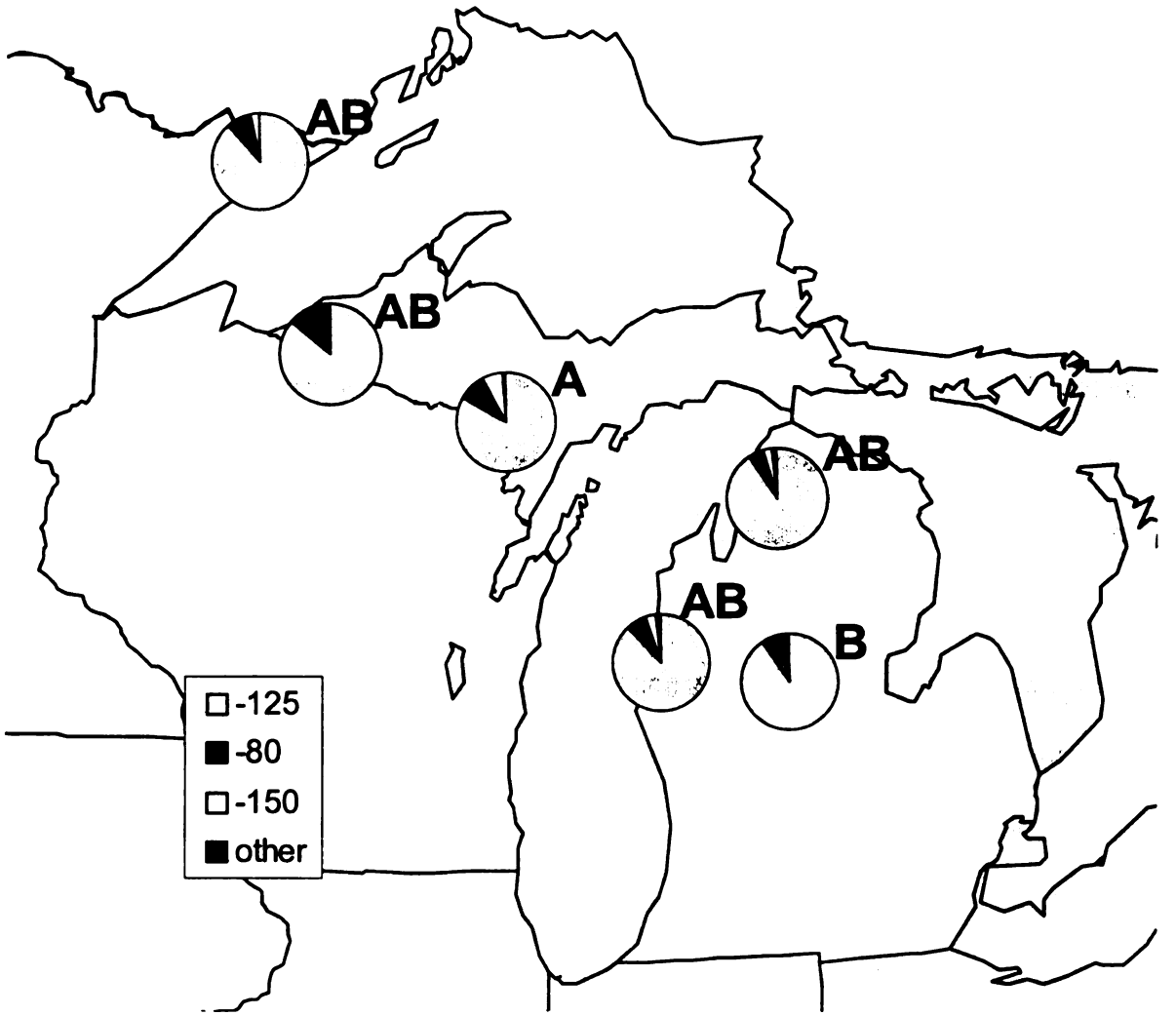


Figure 4.5. PGD allozyme frequencies for six sampled *canadensis* populations. Populations not sharing a letter are significantly different at $p$=0.05. The $P$-value for the test of overall allele differentiation is 0.060.

**Discussion**

I found little evidence of genetic structuring in Great Lakes area *canadensis* populations, which suggests high gene flow between populations. This is in line with many other results with *Papilio* species: high gene flow has been inferred in *P. hospiton* (Aubert et al. 1997), *P. machaon* (Aubert et al. 1997, Hoole et al. 1999), *P. glaucus* (Bossart & Scriber 1995), and *P. zelicaon* (Tong & Shapiro 1989), although isolation has been found between subspecies of *P. troilus* (Margraf & Scriber in prep).

The result of high gene flow within *Papilio* species appears at odds with the local adaptation that is often found (Bossart & Scriber 1995, Tong & Shapiro 1989, Ayres & Scriber 1994). For these and other reasons, inferring gene flow from *F*-statistics based on allozyme data has been criticized (Bossart & Prowell 1998). However, local adaptation need not be inconsistent with high gene flow. If the selection on some character is weak, that selected character can be unlinked from other loci (such as enzyme loci), producing no allele differentiation at most loci with high differentiation at a few (selected) loci. This seems possible for such traits as hostplant use efficiency (immigrant individuals will still be able to survive on the new local host, just with lower efficiency). Still, it is wise to follow Daly (1989), who recommends treating a result of high gene flow inferred from allozymes as a hypothesis of high gene flow, not a concrete conclusion.

These results produce a working hypothesis of high gene flow between *canadensis* populations, even those separated by the Great Lakes. This matches a previous result of high gene flow between *glaucus* populations (Bossart & Scriber 1995). Together, these imply that potential gene flow between the two species could be quite high, which would mean that to produce a hybrid zone as narrow as is found, and to

maintain the differences that are found between species (Hagen et al. 1991), barriers to gene flow between *canadensis* and *glaucus* must be quite strong. However, as yet few strong barriers have been found (see Chapters 2 & 3).

# CHAPTER 5:

# INTROGRESSION OF *PAPILIO GLAUCUS* GENES INTO *P. CANADENSIS* POPULATIONS

## Introduction

Introgression is a special case of gene flow: the passage of alleles from one species to another, which comes about as a result of successful hybridization. There is debate as to the importance of introgression to evolution (Arnold et al. 1999), but regardless of its importance it is informative as to the strength and completeness of barriers to gene flow between species. Hybridization and introgression can be difficult to detect based on morphology, but molecular markers can be very powerful in this respect (Scriber et al. 1995).

Between *Papilio canadensis* and *P. glaucus*, introgression has been detected at all three diagnostic allozyme loci (*Pgd*, *Ldh*, and *Hk*) (Hagen et al. 1991). It is thought to be partially responsible for the appearance of the "spring form" of *glaucus*: *canadensis*-like individuals that appear in early spring *glaucus* populations (Scriber 1990). However, the extent of introgression at other loci is unknown. Studying introgression at mitochondrial genes is of particular interest because it would track maternal inheritance. Recent phylogenetic studies on *Papilio* based on mtDNA gene sequences have found sequence differences between individuals of different species, and these could yield diagnostic mtDNA markers (Sperling 1993).

In some insect species, it has been found that mtDNA introgresses more readily than nuclear genes (Aubert & Solignac 1990, Powell 1983). Mitochondrial DNA in

*Papilio* might follow this pattern, introgressing more readily than nuclear enzyme alleles, or it may be found that there is very limited mtDNA introgression, possibly due to a Haldane effect weeding out female hybrids more strongly than male (Hagen & Scriber 1995).

Hybrid zones are typically characterized by short, steep clines maintained by strong selection, flanked on either side by long tails of introgression (Barton & Hewitt 1985). In Chapter 4 I examined allozyme frequencies for PGD, which has fixed differences for *glaucus* and *canadensis* (Hagen & Scriber 1991). This means that for these populations, introgressed allele frequencies are already known, providing information on the length of tails of introgression for PGD. This can provide the basis for comparisons of introgression of nuclear and cytoplasmic genes. It also allows us to determine if mtDNA introgression tends to be found in individuals that also carry introgressed nuclear genes.

I first used *canadensis* and *glaucus* individuals from a number of different geographic locations to verify the fixation of alternate mtDNA haplotypes as revealed by PCR-RFLP (Polymerase Chain Reaction, followed by Restriction Fragment Length Polymorphism). The resulting diagnostic molecular marker was then used to compare mitochondrial introgression to nuclear introgression at the *Pgd* gene locus in the *canadensis* population samples from Chapter 4, plus a *glaucus* population sampled the same year. Finally I determined if introgression at one gene tended to be coincidental within individuals with introgression at other genes.

**Methods**

Fifteen *canadensis* individuals and seventeen *glaucus* individuals from a number of geographic locations, all collected prior to 1997, some stored at -80°C and others stored as pinned specimens at room temperature (Table 5.1) were used to verify the consistency of the PCR primer sites and the restriction site that was used. DNA extraction methods followed Sperling & Hickey (1995). From each specimen, two legs were plucked and macerated in 800 µl of Lifton buffer (0.2M sucrose, 50mM EDTA, 100mM Tris, and 0.5% SDS). Samples were vortexed and left at room temperature for 30 minutes. Then 100 µl 8M KoAc was added and each sample was inverted and put on ice for 60 minutes. Samples were centrifuged for 20 minutes and the supernatant was transferred to a new tube. Samples were extracted once with phenol and once with chloroform/isoamyl alcohol (24:1). Samples were then precipitated in isopropanol, washed with 70% ethanol, then dried and resuspended in 200 µl 1X TE buffer.

The PCR primers that were used had sequences 5' ATA ATT GGA GGA TTT GGA AAT TG 3' and 5' ATT GTA GTA ATA AAA TTA ATT GCT CC 3', provided by F.A.H. Sperling (University of California, Berkeley). These primers were produced as a result of sequencing work on *canadensis* and *glaucus* mitochondrial COI and COII genes (Caterino & Sperling 1999), and were expected to produce a DNA fragment 294 base pairs long. Within this fragment were five potentially diagnostic restriction sites, also provided by Dr. Sperling. I chose a *Taq*I restriction site anticipated to be present in *glaucus* individuals and absent in *canadensis* individuals.

PCR was carried out using the above primers in a total reaction volume of 100 µl using AmpliTaq Gold DNA polymerase in a Perkin Elmer GeneAmp 9600 Cycler. PCR

products were verified by running them out on a 2% agarose gel along with a 100bp DNA ladder, visualized by ethidium bromide (EtBr) under ultraviolet light. PCR products were then digested by *TaqI* restriction enzyme incubated at 65°C for 120 minutes, and digested DNA was also run out on a 2% EtBr agarose gel with a 100bp DNA ladder for comparison.

To compare cytoplasmic and PGD introgression, the six *canadensis* populations from Chapter 4, plus a *glaucus* population from southern Ohio also sampled in May 1998, were used (Figure 5.1). PGD allozyme determination is described in Chapter 4. The PGD allozymes from the six *canadensis* populations described in that chapter were compared to those determined for the *glaucus* population. Twelve individuals from each of the seven populations were randomly chosen, and for these twelve individuals the mtDNA haplotype (as revealed by *TaqI* PCR-RFLP) was determined. Additionally, all individuals carrying PGD interspecific introgression were haplotyped as well.

**Results**

All but one of the 32 individuals picked to verify PCR-RFLP had successful PCR products (Table 5.1). This included both frozen and dried specimens. The one specimen for which PCR was unsuccessful was a dried *canadensis* specimen. It is unknown if the PCR for this individual was unsuccessful due to degraded DNA, lack of primer correspondence, or an unsuccessful DNA extraction. All other 31 specimens had a PCR product slightly shorter than 300bp long, exactly as long as would be expected based on sequencing. No individual produced two PCR fragments.

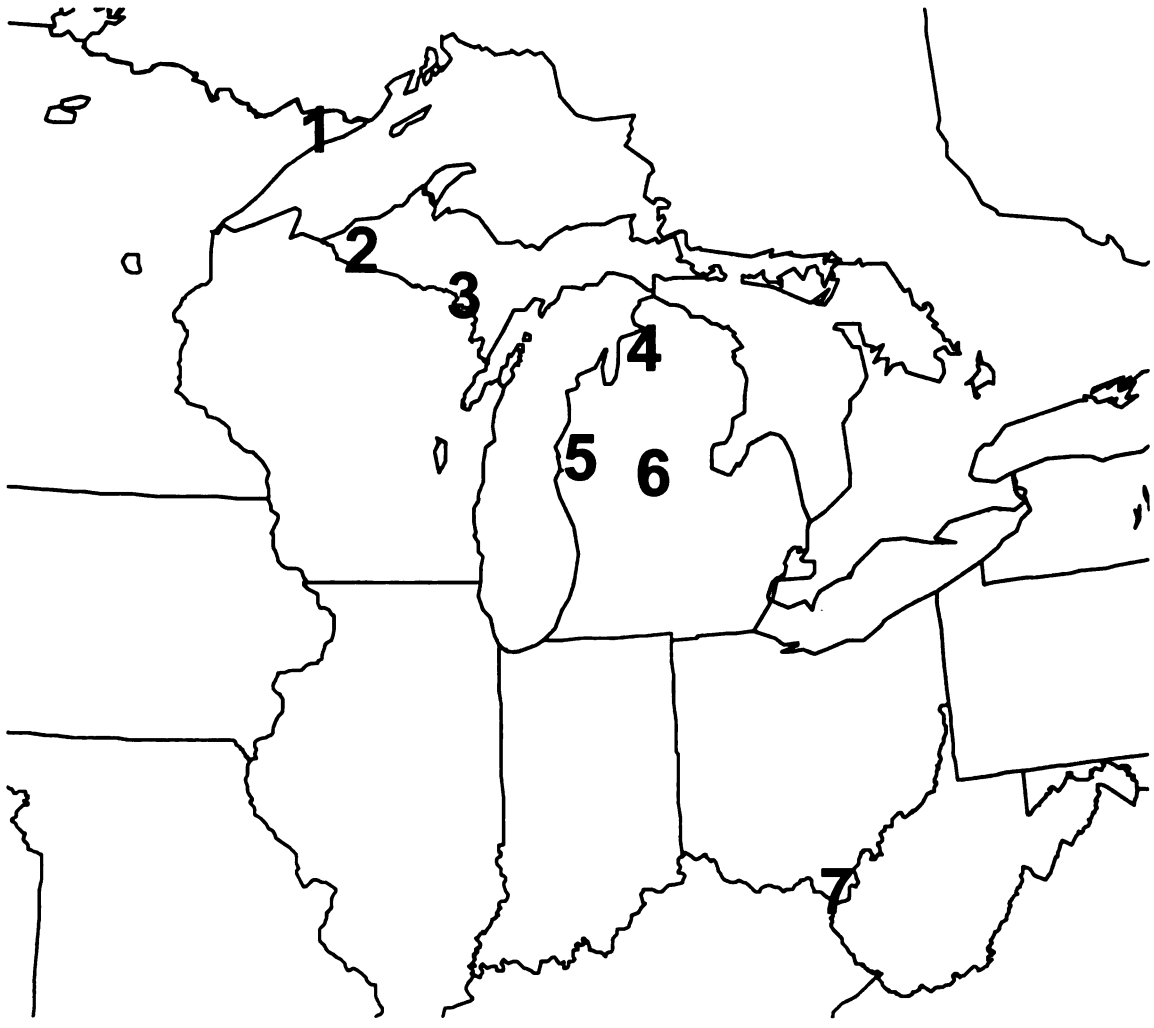


Figure 5.1. Sites of six sampled *canadensis* populations and one sampled *glaucus* population collected in May and June 1998. Sampled *canadensis* populations. 1: Cook Co., Minnesota; 35 males. 2: Gogebic Co., Michigan; 36 males, 1 female. 3: Dickinson Co., Michigan; 48 males, 20 females. 4: Charlevoix Co., Michigan; 50 males, 18 females. 5: Mason Co., Michigan; 50 males, 15 females. 6: Isabella Co., Michigan; 50 males, 14 females. Sampled *glaucus* populations. 7: Lawrence Co., Ohio; 22 males.

Table 5.1. Verification of diagnostic mtDNA haplotypes for *canadensis* and *glaucus* as visualized by PCR-RFLP. Frozen specimens had been stored at -80°C, dried specimens had been stored pinned in drawers at room temperature. The *canadensis* haplotype (–) is indicated by the absence of a *Taq*I restriction site in the 294bp PCR fragment, the *glaucus* haplotype (+) is indicated by the presence of a *Taq*I restriction site in the same fragment.

| Species | Origin | Storage | mtDNA haplotype |
|---|---|---|---|
| *canadensis* ♀ | Fairbanks, Alaska 6/95 | frozen | (–) |
| *canadensis* ♀ | Fairbanks, Alaska 6/95 | frozen | (–) |
| *canadensis* ♂ | Fairbanks, Alaska 6/95 | frozen | (–) |
| *canadensis* ♂ | Fairbanks, Alaska 6/87 | dried | (–) |
| *canadensis* ♀ | Thunder Bay, Ontario 6/95 | frozen | (–) |
| *canadensis* ♂ | Thunder Bay, Ontario 6/95 | frozen | (–) |
| *canadensis* ♀ | Pancake Bay, Ontario 6/95 | frozen | (–) |
| *canadensis* ♂ | Bayfield Co., Wisconsin 6/95 | frozen | (–) |
| *canadensis* ♂ | Forest Co., Wisconsin 6/95 | frozen | (–) |
| *canadensis* ♂ | Lincoln Co., Wisconsin 6/85 | dried | (–) |
| *canadensis* ♂ | Ontonagon Co., Michigan 6/87 | dried | * |
| *canadensis* ♂ | Mackinac Co., Michigan 6/96 | frozen | (–) |
| *canadensis* ♀ | Charlevoix Co., Michigan 6/95 | frozen | (–) |
| *canadensis* ♂ | Manistee Co., Michigan 6/95 | frozen | (–) |
| *canadensis* ♀ | Isabella Co., Michigan 6/96 | frozen | (–) |
| *glaucus* ♀dark | Dane Co., Wisconsin 8/83 | dried | (+) |
| *glaucus* ♀yellow | Dane Co., Wisconsin 8/83 | dried | (+) |
| *glaucus* ♂ | St. Joseph Co., Michigan 7/95 | frozen | (+) |
| *glaucus* ♂ | St. Joseph Co., Michigan 7/95 | frozen | (–) |
| *glaucus* ♂ | Adams Co., Ohio 7/85 | dried | (+) |
| *glaucus* ♀dark | Lawrence Co., Ohio 9/95 | frozen | (+) |
| *glaucus* ♀yellow | Lawrence Co., Ohio 9/95 | frozen | (–) |
| *glaucus* ♂ | Wise Co., Virginia 8/94 | frozen | (+) |
| *glaucus* ♂ | Wise Co., Virginia 8/94 | frozen | (+) |
| *glaucus* ♂ | Clarke Co., Georgia 5/87 | dried | (+) |
| *glaucus* ♂ | Clarke Co., Georgia 8/95 | frozen | (+) |
| *glaucus* ♀dark | Clarke Co., Georgia 8/95 | frozen | (+) |
| *glaucus* ♀yellow | Clarke Co., Georgia 8/95 | frozen | (+) |
| *glaucus* ♂ | Highlands Co., Florida 4/82 | dried | (+) |
| *glaucus* ♂ | Highlands Co., Florida 9/95 | frozen | (+) |
| *glaucus* ♀dark | Highlands Co., Florida 9/95 | frozen | (+) |
| *glaucus* ♀yellow | Highlands Co., Florida 9/95 | frozen | (+) |

* No DNA amplified.

None of these fourteen *canadensis* specimens with successful PCR had a fragment that was cut by the *Taq*I restriction enzyme (Table 5.1). Fifteen of the seventeen *glaucus* specimens had PCR fragments that were cut by the *Taq*I restriction enzyme, producing a fragment slightly longer than 200bp long and another fragment that was not visualized by EtBr (probably because of its size, there is not enough DNA to fluoresce brightly enough under the UV). Two *glaucus* individuals had PCR fragments uncut by *Taq*I. This means that the presence of a *Taq*I restriction site in this DNA region can be taken as a mitochondrial marker for *glaucus*, and the absence of this site can be taken as a marker for *canadensis*.

For the 1998 population samples, relative frequencies of *canadensis* and *glaucus* PGD alleles and mtDNA haplotypes are shown in Figure 5.2. PGD introgression was found in the three lower peninsula *canadensis* populations and in the one *glaucus* population, all at frequency lower than 0.1 (0.018 in Charlevoix Co., 0.009 in Mason Co., 0.035 in Isabella Co., 0.068 in Lawrence Co.). Introgression at mtDNA was found only at Mason and Isabella counties (one out of twelve individuals, 0.083 for both). No introgression was found in either Michigan Upper Peninsula population or the northern Minnesota population.

There were two *canadensis* individuals carrying mtDNA introgression. One carried no introgression at any of the diagnostic allozyme loci (*Pgd*, *Ldh*, *Hk*), and the other carried introgression only at *Hk*, and was heterozygous at that locus (Table 5.2). There were eight individuals with introgressed *Pgd* alleles, and seven of them had no other introgressed alleles at either of the other enzyme loci or in their mtDNA. The eighth carried introgression also at *Hk* (again heterozygous there) but not at the other loci.

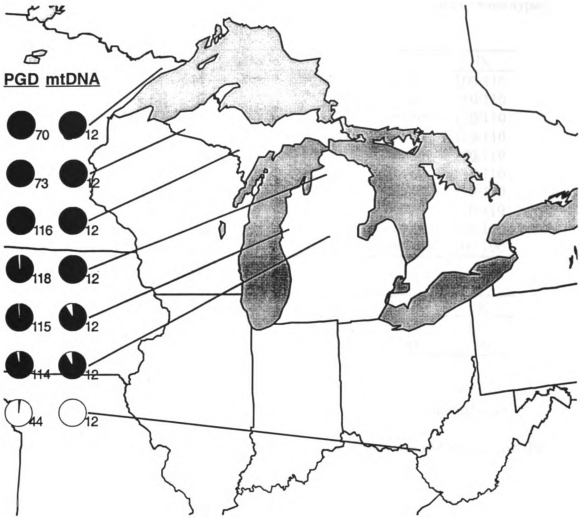


Figure 5.2. Diagnostic molecular marker frequencies for six *canadensis* populations and one *glaucus* population sampled in 1998. Filled portions indicate frequencies of *canadensis* alleles (for PGD) or haplotypes (for mtDNA) and open portions indicate frequencies of *glaucus* alleles or haplotypes. The left column indicates PGD frequencies, the right column mtDNA. Numbers next to pies indicate the numbers of alleles or haplotypes sampled.

Table 5.2. Individuals from 1998 samples carrying introgressed alleles or haplotypes. Introgressed alleles are underlined.

| Individual | mtDNA | PGD | LDH | HK |
|---|---|---|---|---|
| *canadensis* Charlevoix MI ♂44 | (–) | -125/-50 | 80/80 | 100/110 |
| *canadensis* Charlevoix MI ♀10 | (–) | -100 | 80 | 110/110 |
| *canadensis* Mason MI ♂2 | (–) | -125/-100 | 80/80 | 110/110 |
| *canadensis* Mason MI ♂21 | (+) | -125/-125 | 80/80 | 110/110 |
| *canadensis* Isabella MI ♂5 | (+) | -125/-125 | 80/80 | 100/110 |
| *canadensis* Isabella MI ♂13 | (–) | -125/-100 | 80/80 | 110/110 |
| *canadensis* Isabella MI ♂43 | (–) | -125/-100 | 80/80 | 110/110 |
| *canadensis* Isabella MI ♀10 | (–) | -100 | 80 | 110/110 |
| *canadensis* Isabella MI ♀13 | (–) | -100 | 40 | 110/110 |
| *glaucus* Lawrence OH ♂13 | (+) | -125/-100 | 100/100 | 100/100 |

Key

| Species | mtDNA | PGD | LDH | HK |
|---|---|---|---|---|
| *canadensis* | (–) | -125 | 80, 40 | 110 |
| *glaucus* | (+) | -50, -100 | 100 | 100 |

Within individuals, introgression at one locus was usually not coincidental with introgression at other loci. This means that this introgression was old, rather than the result of primary hybridization.

**Discussion**

I found the DNA extractions and PCR reactions to be quite reliable, even when using small amounts of tissue (from plucked legs) and specimens that had been dried and stored at room temperature for over twelve years. The *Taq*I restriction site was found to be almost absent in *canadensis* populations and almost fixed in *glaucus* populations (Table 5.1, Figure 5.2). Individuals of one species carrying the haplotype of the other were found in both species. This can still represent a diagnostic character, because 1)

such individuals were rare, and 2) they had been collected from areas near hybrid zone areas, so these cases could be explained as introgression.

PGD introgression was found in three *canadensis* populations and in one *glaucus* population sampled in 1998. Because of the high sample numbers for those populations (44 alleles sampled for the *glaucus* population, over 100 alleles sampled for these *canadensis* populations), the frequencies determined here are probably indicative of what they were in the wild, and PGD introgression in these populations for that year is concluded to have been present at low frequencies.

In the 1998 sample, mtDNA introgression was only found in the two southernmost *canadensis* populations. However, because of the sample sizes (only twelve haplotypes sampled for each population) the actual frequencies in the populations cannot be estimated with confidence. Introgression at mtDNA might be at higher frequency than at PGD in these populations, but a larger sample would be needed to determine this. Although no introgressed mtDNA was found in the *glaucus* population in the 1998 sample, it was found in two *glaucus* individuals in the initial survey: one individual was from St. Joseph County in Michigan (which is near the hybrid zone), the other was from Lawrence County in Ohio (where no mtDNA introgression was found in 1998). Southern Ohio is quite far from the Michigan hybrid zone, but it is near a tail of *canadensis* hybridization that extends southward along the Appalachian mountain range.

Introgressed alleles (both in *Pgd* and in mtDNA) in the 1998 *canadensis* populations were only found in the lower peninsula populations (Figure 5.2). This is evidence for genetic structure between *canadensis* populations: some reduction in gene flow between populations. However, the genetic structure is slight (because introgressed

alleles are found at low frequency). This means that the small $F_{ST}$-values found in Chapter 4 cannot be interpreted as being equal to zero. There is some small reduction in gene flow between populations. However, gene flow between populations is still quite strong.

Within individuals, introgression at either mtDNA or *Pgd* was generally not coincidental with introgression at other loci (Table 5.2). This indicates that most introgression was not recent, giving time to separate loci. The introgressed alleles now may be under negative selection, or they may be merely acting like any other rare alleles.

If introgressed molecular markers are typically noncoincidental within individuals, then probably introgressed ecological characters (diapause, oviposition preference, host use ability) and morphological characters (size, wing morphometrics, laraval characters) will also have become unlinked to other introgressed characters. This means that if an individual in a *canadensis* population such as Isabella or Charlevoix Counties is found with *glaucus*-like oviposition preference or host use ability, there is no reason to expect to find other *glaucus*-like characters.

The presence of introgressed PGD alleles and mtDNA haplotypes indicates that barriers to gene flow are not complete. However, because it is limited in both frequency and distance from interspecific populations, the barriers to gene flow that are present must be quite strong.

# CHAPTER 6:

# SUMMARY AND CONCLUSIONS

I found evidence from allozymes of high gene flow between species of *canadensis* through the Great Lakes region (Chapter 4), matching a previous result showing high gene flow in *glaucus* (Bossart & Scriber 1995). Introgression of *glaucus* genes into *canadensis* populations and vice-versa was found at both nuclear and mitochondrial loci (Chapter 5). Mitochondrial introgression indicates that some introgression is female-mediated, despite potential Haldane effects against female hybrids (Hagen & Scriber 1995). However, introgression was very limited, both in frequency within populations and in the length of the tails of introgression.

High gene flow and limited introgression indicates that there must be either strong barriers to gene flow between species or strong selection against hybrids. So what maintains the species differences between *canadensis* and *glaucus*?

There does not appear to be postpairing, prezygotic isolation, at least once mates have locked together (Chapters 2 & 3). However, it would be worthwhile to investigate mate recognition very early in the mating, before locking occurs. Some pairings separate within a couple minutes of the start of the pairing, and if heterospecific pairings do so more often than conspecific, hybrid production would be reduced. This isolation would fit with the content of Chapter 2, but it would be difficult to study in the lab using hand-pairings. Rather, because such isolation would probably be strongly affected by behavior, it would be better to study naturally initiated matings occurring in more natural conditions.

The other aspect of prezygotic isolation that has not yet been addressed with these species is female choice during courtship. Male choice has been studied (it might be an important barrier in *glaucus*, but it would appear to increase hybridization into *canadensis* populations, Deering 1998), and it makes sense to do so in *Papilio* because of the costliness of male ejaculates (Gwynne 1984, Lederhouse 1995). However, females should still be the more discriminating of the two (Darwin 1871), and it has been observed that *glaucus* females are able to spurn potential (conspecific) male mates (Krebs 1988). Studying female choice of conspecific males versus heterospecific males should be very important for understanding maintenance of species differences. Intraspecific mate choice of *Papilio glaucus* females has been studied in large flight cages by Krebs (1988), and this approach could be used to study interspecific mate choice as well.

Endogenous selection against hybrids of these species appears to be weak (Hagen & Scriber 1995). Hybrids are viable and fertile, and the only Haldane effect so far identified is a slightly higher mortality of *glaucus* × *canadensis* female pupae (Hagen & Scriber 1995). Chapter 2 found that *canadensis* × *glaucus* hybrid females pair with success equal to pure species *canadensis* females paired to conspecific males. This is a measure of hybrid fitness not previously studied in *Papilio*. However, hybrid breakdown (endogenous weakness of backcross individuals or F2 hybrids) remains incompletely studied.

Ecological selection against hybrids is another potential barrier to gene flow (Sperling 1990). Diapause might be critical: because the *canadensis* obligate diapause gene is recessive, most individuals in a *canadensis* population introgressed for that gene will not diapause, which would likely be a fatal error in a time-limited growing season.

In *glaucus* populations (which normally undergo two or more flights per year), entering diapause after the first flight could leave the resulting pupa open to increased predation or parasitism for the remainder of the summer (West & Hazel 1982). There are a number of other ecological factors of potential interest: 1) are hybrids sexually attractive; do host use abilities break down upon backcrossing and other crossing; and are the diagnostic allozyme loci actually adapted to their respective ranges? Exogenous selection against hybrids could come in the form of weak selection on a combination of these traits.

There are many important ecological and evolutionary aspects to the study of hybrid zones (Harrison 1993, Howard & Berlocher 1998). One central area of interest, which was a focus of this thesis, is the identification of the barriers to gene flow that maintain differences across clines. Another is the distribution of traits diagnostic for the two species, and how genes for these traits move within and between populations, another focus of this thesis. These two swallowtail butterflies provide an excellent example for the study of the maintenance of species differences across hybrid zones. This story is especially interesting because of the intriguing behavior of the males (Deering 1998), and the high fitness of the hybrids (Hagen & Scriber 1995). Another advantage this system offers is the number of ecologically important differences between these species that have been identified (Scriber et al. in press). Continued study of potential barriers to gene flow between *canadensis* and *glaucus*, as well as of clines of multiple traits, as was done with PGD and mitochondrial DNA in this thesis, will continue to improve our knowledge of this unique system.

# APPENDICES

# APPENDIX 1:

# RECORD OF DEPOSITION OF VOUCHER SPECIMENS

## APPENDIX 1

### Record of Deposition of Voucher Specimens*

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

Voucher No.: 2000-05

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

Lack of cryptic reproductive isolation between Papilio canadensis and Papilio glaucus; and population genetics near their hybrid zone

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

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name (s) (typed)
Aram Daniel Stump

Date 21 August 2000

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

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

## APPENDIX 1.1:

## VOUCHER SPECIMEN DATA

APPENDIX 1.1

Voucher Specimen Data

Page 1 of 4 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. | GEORGIA Clarke Co.<br>18 August 1997<br>J. Maudsley | | | | | | 1 | | MSU |
| | Lab-reared Brood 13188<br>Maternal Origin:<br>OHIO Lawrence Co.<br>20 August 1997<br>M. Deering | | | | | | 1 | | MSU |
| | Lab-reared Brood 13119<br>Maternal Origin:<br>OHIO Lawrence Co.<br>10 August 1997<br>W. Wehling | | | | | 1 | | | MSU |
| | Lab-reared Brood 12416<br>Maternal Origin:<br>OHIO Lawrence Co.<br>30 July 1996<br>M. Deering | | | | | 1 | | | MSU |

(Use additional sheets if necessary)

Investigator's Name(s) (typed)
Aram Daniel Stump

Date 21 August 2000

Voucher No. 2000-05
Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator 21 August 2000
Date

APPENDIX 1.1

Voucher Specimen Data

Page 2 of 4 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 multicaudatus Kirby | Colorado Jefferson Co.<br>28 July 1997<br>H.F. Wehling | | | | | 2 | 1 | | MSU |
| | California Siskiyou Co.<br>Shasta River<br>27 May 1995<br>D. McCorkle | | | | | | 1 | | MSU |
| Papilio troilus L. | Michigan St. Joseph Co.<br>Three Rivers Game Area<br>30 July 1999 | | | | | | 2 | | MSU |
| | Lab-reared Brood 14312<br>Maternal Origin:<br>Missouri St Charles Co.<br>21 July 1998 | | | | | 1 | | | MSU |
| | Lab-reared Brood 14308<br>Maternal Origin:<br>Missouri St Charles Co.<br>21 July 1098<br>M. Deering | | | | | 1 | | | MSU |

(Use additional sheets if necessary)

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Aram Daniel Stump

Date 21 August 2000

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

Curator Date

**APPENDIX 1.1**

**Voucher Specimen Data**

**Page 3 of 4 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 eurymedon Lucas | California El Dorado Co.<br>Highway 50<br>24 July 1998<br>R. Dowell | | | | | 1 | 2 | | MSU |
| | California El Dorado Co.<br>29 July 1999<br>R. Dowell | | | | | | 1 | | MSU |
| | Lab-reared Brood 11320<br>Maternal Origin:<br>California Mono Co.<br>Sonora Pass<br>6 July 1995<br>R. Dowell | | | | | | 1 | | MSU |
| | Oregon Polk Co.<br>Monmouth<br>August 1995<br>D. McCorkle | | | | | | 1 | | MSU |

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Aram Daniel Stump

Date 21 August 2000

Voucher No. 2000-05

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

Curator Date

APPENDIX 1.1

Voucher Specimen Data

Page 4 of 4 Pages

| Species or other taxon | Label data for specimens collected or used and deposited | Number of: Eggs | Larvae | Nymphs | Pupae | Adults ♀ | Adults ♂ | Other | Museum where deposited |
|---|---|---|---|---|---|---|---|---|---|
| Papilio canadensis Rothschild & Jordan | Lab-reared Brood 12154<br>Maternal Origin:<br>MICHIGAN Isabella Co.<br>16 June 1996<br>J.M. Scriber | | | | | | 1 | | MSU |
| | Lab-reared Brood P133<br>Maternal Origin:<br>MICHIGAN Mackinac Co.<br>Caffey Cemetery<br>14 June 1996<br>P. Giroux | | | | | | 1 | | MSU |
| | Lab-reared Brood 12303<br>Maternal Origin:<br>MICHIGAN Ontonagon Co.<br>3 July 1996<br>W. Wehling | | | | | 1 | | | MSU |
| | Lab-reared Brood P114<br>Maternal Origin:<br>MICHIGAN Emmet Co.<br>Red School Road<br>12 June 1996<br>P. Giroux | | | | | 1 | | | MSU |

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Aram Daniel Stump

Date 21 August 2000

Voucher No. 2000-05

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

Curator Date

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## LITERATURE CITED


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