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Preferential mate selection by males as a reproductive
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PREFERENTIAL MATE SELECTION BY MALES AS A REPRODUCTIVE ISOLATING MECHANISM BETWEEN THE SWALLOWTAIL SPECIES; *PAPILIO GLAUCUS* AND *P. CANADENSIS* (LEPIDOPTERA, PAPILIONIDAE)

By

Mark David Deering

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ABSTRACT

PREFERENTIAL MATE SELECTION BY MALES AS A REPRODUCTIVE ISOLATING MECHANISM BETWEEN THE SWALLOWTAIL SPECIES; *PAPILIO GLAUCUS* AND *P. CANADENSIS* (LEPIDOPTERA, PAPILIONIDAE)

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Papilio glaucus and *P. canadensis* (Lepidoptera: Papilionidae) in the United States have a narrow hybrid zone between them. Hybrids of both species are viable and host plants are found on either side of the hybrid zone, so interspecific mating would be expected to extend the hybrid zone. Mate preference as a pre-zygotic isolating mechanism may account for the narrowness of the zone. Research conducted in Florida and Michigan attempted to determine the importance of such a mechanism. In Florida, 1997, *P. glaucus* males chose conspecific, yellow females for copulation 94.8% of the time (n=69), choosing heterospecific mates only 5.2%. In 1998 males chose *P. glaucus* females in all cases (n=30). In Northern Michigan *P. canadensis* males did not chose conspecific females. Rather, of 493 copulations recorded in 1997 (21 pair combinations) heterospecific *P. glaucus* females were chosen 82.3%. Mate choice by males could play a large role in gene flow outside the hybrid zone.

Dedication

This work is dedicated to my Mother and Father, who never gave up on me.

ACKNOWLEDGMENTS

There are many people who helped to make this project possible, and I would like to recognize a few of them. Firstly, my beautiful and supporting wife, who helped me throughout this endeavor. Dr. J. Mark Scriber, my advisor and friend for his many hours of help and dedication to making sure that this project succeeded. I would like to thank the many graduate and undergraduates who helped in the lab raising stock for this project as well as offering moral support. I thank the department of Entomology whose faculty and staff were ever supportive and helpful with problems and solutions.

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Introduction

It is known that a hybrid zone exists between the two closely related swallowtail species *Papilio glaucus* and *P. canadensis* (Rothschild & Jordan 1906). Rothschild and Jordan (1906) first described *P. canadensis* as a sub-species of *P. glaucus*, and it has since been designated as a species (Hagen et al. 1991). The hybrid zone, which corresponds closely with the boreal/temperate plant tension zone (Hagen 1990, Scriber & Gage 1995), has been delineated well by researchers using adult wing morphology of *P. glaucus* and *P. canadensis* (Luebke et al. 1988), electrophoresis (Hagen & Scriber 1989) as well as other diagnostic traits (Scriber 1996, Hagen et al. 1991). The hybrid zone is narrow across most areas of contact between the two species, though it broadens across the Appalachian range in New York, due to the geographic overlap of different habitats and temperature zones caused by the altitude differences throughout that area (Scriber 1996). Gene flow across the Wisconsin and Michigan areas of the zone appears to be especially limited (Luebke et al 1988, figure 1). This may be due in part to the lack of suitable forest edge and hedgerow habitat in this area, but also may be due to other biotic and abiotic isolating mechanisms. The narrowness of the zone through this area is not unexpected as it corresponds to the

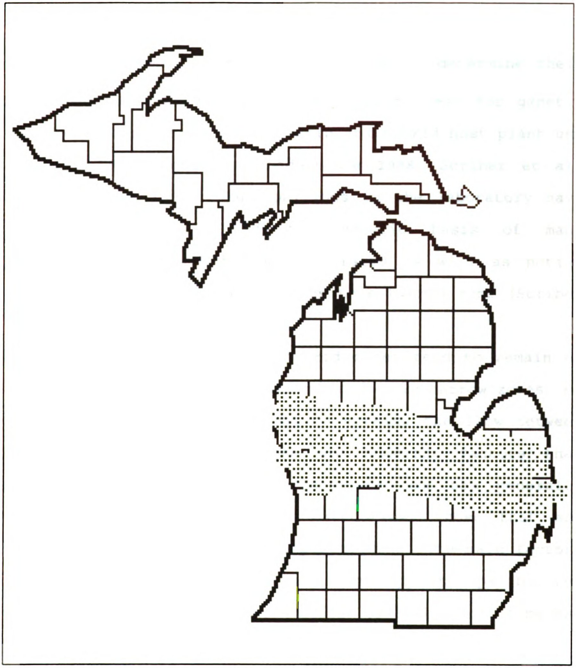


Figure 1: The Hybrid Zone in Michigan.

transition zone between the plant ecotones (Remington 1968). Other *Papilio* species have been studied to determine their interspecific hybrid compatibility and to test for genetic differences in larval survivorship and hybrid host plant use (Scriber 1996, Scriber & Lederhouse 1988, Scriber et al. 1988, 1991, & 1995). Hand pairings in the laboratory have allowed investigation of the genetic basis of many previously unstudied ecological traits as well as noting diagnostic genetic markers across this hybrid zone (Scriber 1996).

Most animals that form hybrid zones tend to remain as separate and distinct populations with only narrow zones, or isolated pockets of hybridization marking the flow between them (Harrison, 1993). There are numerous factors that facilitate this separation. These factors are generally separated into two different categories; those that are prezygotic and those that are postzygotic. The main factors in the prezygotic category are sperm/egg incompatibility, mate recognition and mate choice by males or females, mechanical isolation (lock and key hypothesis) (Porter & Shapiro 1990) and sperm precedence/manipulation (cryptic sexual selection). Major factors in the post-zygotic category are egg inviability, low fecundity and larval survivorship, F1 hybrid infertility or unfitness, and the Haldane effect. The Haldane effect, which causes the death of the F1 females, or the F1a and F2 hybrid females' ova or young larvae before they have a chance to develop and reproduce (Hagen & Scriber 1995), has been well studied by researchers (Coyne et al. 1991, Sperling 1993, Coyne 1994).

This study focuses on male mate choice among paired, tethered, size-matched females of *P. canadensis* and *P. glaucus*. Strong preferences for conspecific females may act as a reproductive isolating mechanism responsible for restricting gene flow across the hybrid zone. Free flying *P. canadensis* males in Northern Michigan and free flying *P. glaucus* males in Central Florida were bioassayed to determine their mating preferences (i.e. for the conspecific or heterospecific female of the pair). Active female solicitation flights have been observed in *P. glaucus* (Krebs & West, 1988), therefore female mate choice could certainly be considered as a possible factor in this type of mating system (Rutowski 1984). However, in the field it has been observed that males of *P. glaucus* or *P. canadensis* initiate courtship flight and behavior. Therefore, my research was focused on this primary component of mate selection by using size-matched, tethered pairs of females placed in male flyways in natural populations. If species-specific assortative mate responses are in effect (Mayr, 1963) then strong preferences for the conspecific of the pair would be predicted, both for *P. glaucus* populations in Florida and for *P. canadensis* populations in Northern Michigan. However, random mating could result if these populations are far from the hybrid zone where reinforcement of correct choice might be relaxed due to the rarity of encounters with the heterospecific female (Howard 1993, Butlin 1995). Figure 2 illustrates the patterns of preference that might be noted if reinforcement/reproductive character displacement or a specific mate recognition system (SMRS) is in effect.

Tethering live butterflies in field experimentation was first utilized for determining mate recognition and choice in some western species of *Papilio* (Brower 1959) and has since been used in only a few studies. Examples of these include one dealing with mate preference in *Papilio glaucus* (Lederhouse 1995), and one dealing with lizard predation on butterflies (Boyden 1976). Tethering live insects in the field allows for a more natural and realistic approach to observing the behaviors of live insects as compared to many laboratory experiments. The courtships and copulations in *Papilio* species often take place very quickly and usually involve only one female, this is a no choice situation. Tethering pairs of heterospecific females provides a way to determine if there is mate preference shown by males for one or the other female in a pair.

Much of the data available regarding the genetic basis of reproductive isolation are of the postzygotic type, and it is frequently assumed that any hybrids produced will invariably be of lower vigor than either of the progenitors (Mayr 1963, Wagner 1970, White 1978, King 1993, but see also

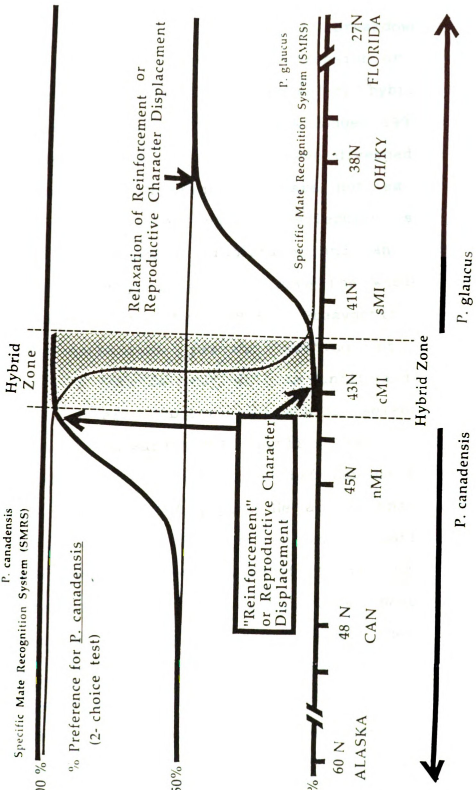


FIGURE 2: EXPECTED PATTERNS OF MATE CHOICE FROM TWO MATE CHOICE SYSTEMS

Arnold & Hodges 1995). The eventual breakdown of hybrid zones may result from neutral introgression or selection of alleles that may reduce the degree of hybrid unfitness (Riesenberg & Wendel 1993, Arnold & Hodges 1995, Ritchie & Hewitt 1995). If two populations have diverged genetically and are producing hybrids that are not as fit as the original species then natural selection should favor prezygotic reproductive isolation and an increase in assortative mating, helping to move the whole population towards speciation (Mayr 1963). Prezygotic reproductive isolating mechanisms prevent wasting of gametes on production of hybrids that will be unfit and unlikely to survive, and are subject to natural selection through reinforcement (Howard 1993, Butlin 1995). Reinforcement across hybrid zones is an idea that can be attributed to Dobzhansky in his 1940 paper. He argued that as the two populations of a species evolved in allopatry that they would also evolve different ways of recognizing their mates, both behaviorally and genetically. Once these populations were back in contact with each other then the hybrid offspring that were produced would suffer. Reinforcement itself would allow for more effective mate recognition in the areas where the populations actually meet and overlap.

> "Thus prezygotic isolating barriers evolve after the adaptive divergence of the incipient species, and this evolution occurs primarily in areas where the two taxa overlap in distribution" (Howard 1993).

Reinforcement in mate choice takes place as selection pressures become greater for one or the other of a set of potential mates. This could be due to perceived mate preference (ornamentation) or due to lower survival of offspring with one or the other potential mate, leaving more offspring that may prefer the particular traits of their parents. The idea of prezygotic isolating mechanisms and reinforcement lacks convincing examples, but this is an extremely appealing concept and one that this study attempts to examine. Hopefully other studies will follow as reinforcement and reproductive isolating mechanisms are ideas that deserve more critical research focus (Howard 1993, Butlin 1995, Futuyma and Shapiro 1995, Veech et al. 1996). Rather than using inference and indirect methods, this study used direct observations of assortative mating at different field locations across both species' home ranges to test hypotheses about prezygotic isolating mechanisms.

Materials and Methods

Stock Acquisition and Husbandry

The swallowtails used in these experiments were laboratory reared from stock collected from natural populations. *Papilio glaucus* females were collected from three sites over a three year period. 1) Kentucky (Daniel Boone National Forest). 2) Lawrence County Ohio (Wayne national Forest and Dean State Forest). 3) Clarke County Georgia. *Papilio canadensis* females were collected from wild populations in the Lower and Upper peninsulas of Michigan; primarily Chippewa, Cheboygan, Charlevoix and Otsego counties.

Collected females were processed by measuring the fore-wing length (distance in mm from basal attachment to wing tip), recording the County and area of capture, and issuing the female a "Mother Number" which identified the future offspring as well. For oviposition, females were housed in clear round plastic dishes (12" diameter by 4" deep) and were placed on a turntable which rotated the dishes once every 6 minutes. The insects were positioned on the turntables in front of incandescent lamps and leaves of host plant were inserted into water-filled, rubber-capped, plastic tubes called aquapics (available from florists). Aquapics with host leaves were placed along the inside of the dish for oviposition. Each female was fed once a day on a 20% honey and water solution by drawing their proboscises into a spoon holding the solution. Females readily took food in this manner and some females lived for as long as three weeks. When possible, females were frozen (at -80° C) when near death for later genetic and enzyme analysis.

Eggs from these females were counted and collected daily and new leaves were also added at this time. The new eggs were placed into 110 mm Petri dishes which were then placed in growth chambers set at 25° C. Eggs generally hatched within 3-6 days and were checked daily for newly hatched larvae which were then moved to leaves of fresh host plant in new 110 mm dishes. Leaves of the preferred host plant were kept turgid by inserting freshly cut stems into aquapics. Care was taken not to damage the larvae since they are very easily wounded at this stage. A soft rolling motion with a fine, damp camel hair brush worked well. Branches of host used to feed the larvae were collected from sites in Ingham County Michigan, or were from plants raised in pots in a greenhouse. Branches of host were pruned from near the tops or on the sides of young trees to ensure a good quality diet. The cut ends were placed in water jugs which were then refrigerated. Placement of a plastic bag around the foliage helped to retain moisture while under refrigeration. Foliage was harvested every other day to ensure that the host material was fresh.

Larvae were identified and recorded by mother number and host plant identification abbreviation. For the *P.*

glaucus used in this experiment they were raised on Black Cherry (*Prunus serotina*). *P. canadensis* was also reared on Black Cherry. Since both *P. glaucus* and *P. canadensis* were raised on Black Cherry this removes any chance the host plant differences could play a role in choice by males. Also noted on the dish were the phenotype (species, and color morph if *P. glaucus*), state of origin, date that larvae hatched, and the number of larvae being placed in the dish. The dishes were kept relatively dry by placement of a paper towel circle in the bottom of each dish. Food was changed every 1-2 days, or as needed, by gently removing the larvae from the old leaves and replacing them with fresh ones. Clean, dry paper towel was added at this time and the aquapics were refilled. The number of larvae placed in new dishes ranged from 3-5 depending on available space in growth chambers. Larvae, in their new dishes, were then placed into growth chambers set at 25° C and 1 of 2 photo phases. Larvae of *P. glaucus* reared under a 18:6 L:D period generally developed directly to adults upon pupation. Those larvae reared under a 12:12 L:D period were induced to diapause (Rockey et al. 1987, Tidwell 1995, Valella and Scriber 1998). The pupae of *P. canadensis* require a diapause period regardless of photoperiod.

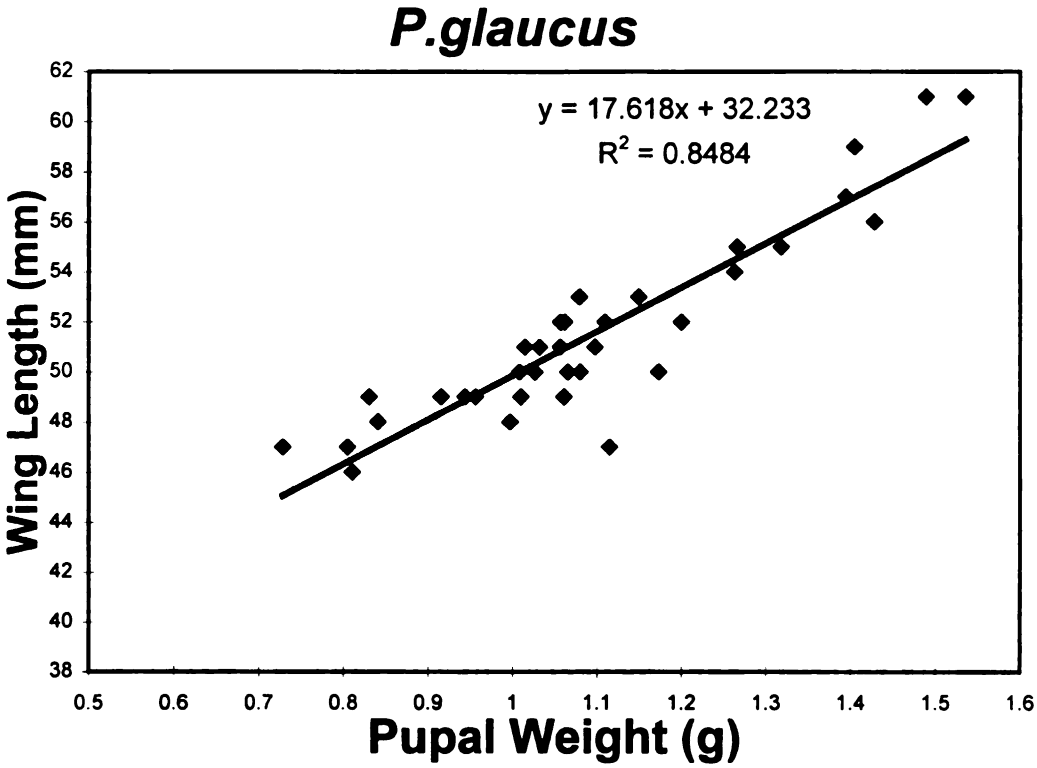
The larvae were separated to 1-2 per dish in the third instar. This was done to try to reduce the amount of disease seen in the cultures. It was suspected that some instances of disease were caused by overcrowding and subsequent nutritional stress. Four or five third instar larvae in a dish can eat all of the host leaves added in less than 24 hours. Disease was a serious factor in larval survivorship, thus care was taken to help minimize disease. A 20% chlorine bleach solution was used to clean countertops, scissors, brushes, branch clippers and in some instances the host leaves themselves. Host leaves would be dipped for 3 minutes into a 5% solution of bleach and water, then rinsed under running water for 10 minutes. This was only done during periods when disease and death among the larvae were very high (above 65%). It appeared that the sanitation methodology used was at least somewhat effective in decreasing the number of larvae dying from disease. Extended exposure to ultra-violet light was also used at regular intervals for the sanitation of tools and growth chambers.

Larvae generally required 20-35 days to develop to pupation at 25° C at either photoperiod (Tidwell 1995). Upon pupation they were removed from the dishes or twigs and the pupae were weighed and sexed. The weights were recorded and the pupae were then placed back into their dish. When the pupae were near eclosion a 10 cm tall cylinder of window screen was placed into the dish so the adults could crawl upwards to hang and allow their wings to expand properly. Those pupae that had been induced to diapause for the winter were placed on the shelf until October. At that time they were placed into growth chambers cooled to 5° C until needed.

Pupae of *P. glaucus* and *P. canadensis* were matched together on the basis of size. Pupae within 0.1g of each other often resulted in adults with wing lengths that were very similar (Figure 3). Removal of pupal stock for experiments took place 8-15 days before the adults were needed. *P. canadensis* females often emerged after only 8-14 days while *P. glaucus* pupae generally took 1-3 days longer. Upon emergence females were placed into Glassine envelopes and refrigerated until needed. Forewing lengths, date of emergence, and the data from the dish were recorded on this envelope. Refrigerated females were fed every other day until they were needed. Timing was very important in the removal of stock for the flights. *P. canadensis* has only one brood a year, usually in early to mid June. Pupae have to be removed approximately 15 days before the flights of both species to ensure sufficient numbers for tethering, some females do not emerge well and some die. Other pairs were removed in smaller batches closer to the flight time to ensure that there were fresh females for use during the later portions of the flight.

Site Choice for Male Mate Preference Studies

Sites for tethering paired females were chosen using the following criteria. Sites chosen had large numbers of



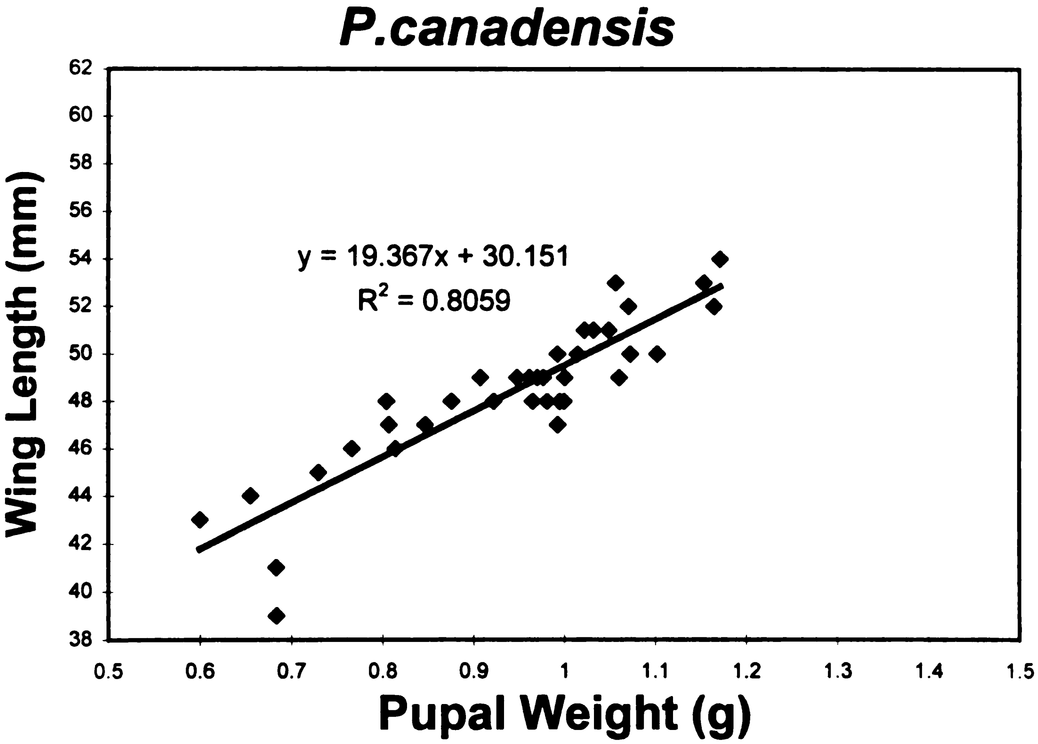


Figure 3: wing length and pupal weight for the two *Papilio* species studied

males in the area, often due to the abundance of nectar sources or because the area was a natural fly-way used by patrolling males. These sites were often along stream edges, hedgerows or roadsides in the North, and along canals or roadsides through the hammock areas in Florida. Sites chosen had shrubs or small tress located in direct sunlight. These were required for the positioning of tethered females. Mobile stakes, branches with "Y" forks on the top for positioning females were used to facilitate placement in the sun or male flyways when bushes or other spots were unavailable.

The site utilized in Florida was Highlands Hammock State Park. This site was chosen mainly because of host plant distribution. The main larval host plant in Florida for *P. glaucus* is Sweet Bay (*Magnolia virginiana*) and these trees were only abundant in the hammock areas of Florida. Experiments were performed at this site in March and April of 1997 and 1998.

The two sites chosen for tethering in Northern Michigan were picked based on population size (visual determination) and nectar sources in the area, primarily lilac (*Syringa vulgaris)* and honeysuckle (*Lonicera sp.*). The first site, used in June of 1996, was in Charlevoix County at Thumb Lake, where males were in abundance on numerous honeysuckle bushes. The second site, used in June of 1997, was also in Charlevoix County at the old Civilian Conservation Corps campsite on C.C.C. road. The population in 1997 at this site was extremely large, with up to 100 *P. canadensis* visible at any time nectaring on the numerous large lilac shrubs in the area.

Female Processing and Tethering

Female imagoes and developing pupae of both *P. glaucus* and *P.canadensis* were taken to the field sites for preparation. Forewing lengths were measured on each of the females. The wing lengths of paired females had to be within 3 mm of each other to be considered for a pairing. Deviations greater than this were considered unacceptable for this experiment. Generally the lengths were identical or at most within 2 mm of each other. The females were carefully size-matched this way due to preliminary data collected in April and May of 1998 showing that Florida males had a decided preference for the larger female of a tethered pair (Scriber and Deering, unpublished). Females were tethered using a 30 cm length of fine black thread tied around the thorax between the wings (Figure 4). The thoracic placement allowed for some slack in the knotted loop which reduced the number of inadvertent dissections as well as allowing the female a more natural range of movements and perching positions. The thread was tied to an alligator clip which was then attached to small branches or twigs on suitable perches. Pairs were tethered approximately 1 meter apart, and approximately 1.5 meters off the ground. Shrubs

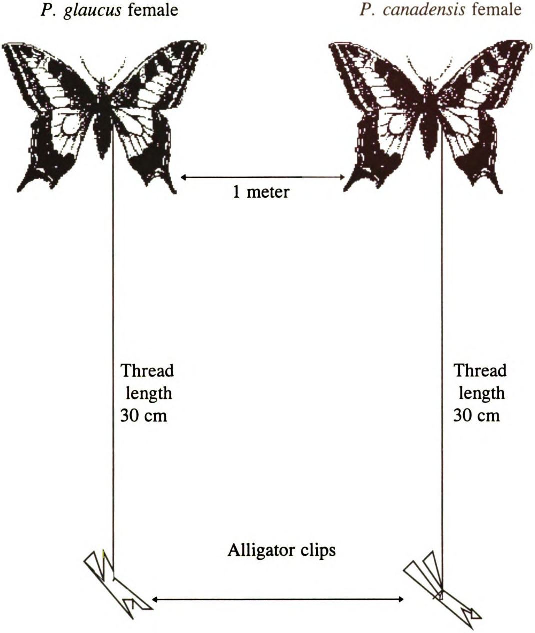


Figure 4: Tethering arrangement for female butterflies in mate choice experiments.

and branches used for tethering were in direct sunlight and were not more than 8 feet tall. The field of view from the perches was at least 180 degrees. Branches that blocked the sight of the females from incoming males were removed prior to the start of the experiment. Females generally fluttered for a short time and then alighted on a perch and rested with their wings spread.

The Experiments

Heterospecific females were tethered side by side for 30 min intervals, then switched to account for different spatial presentations. The pairs were run for two hours, divided into four 30 min bouts, so each female spent 1 h on each of the 2 arrangements. One person was able to tether 2-3 pairs at a time depending on the amount of male activity in the area.

Data was collected and analyzed in three categories:

1) **Touches and flutters:** In this category, males were recorded as they came into contact with the female. This consisted of males fluttering over or under the female for a short time and contacting her at least once.

2) **Attempted matings**: In this category males were recorded if they were very aggressive in grabbing the female or coming in contact with her several times. This was noted when the male captured the female and attempted to initiate

copulation unsuccessfully, sometimes due to interference by other males.

3) Matings: This was recorded when a copulation occurred. Copulations were stopped soon after they started by the gentle removal of the male. Care was taken not to damage the female as this would render her unfit for further experiments. Since males do not pass spermatophores immediately upon mating, often taking 45 minutes or more (personal observation), the females were still considered to be virgin. However, females were usually only used for a total of only 4 hours (2 bouts) and were then retired. Retired females were pinned and mounted so later morphometric or photographic information could be taken.

During the period of tethering the weather, approximate wind speed, approximate temperature and cloud cover were noted. The experiments were done on days with full sun and temperatures above 25 degrees C.. Wind speeds varied considerably but little flight was noted during very strong winds (>30 km/hr).

Analysis

Male preference was determined both for individual pairs and from the population standpoint by analysis of the total number of copulations tallied for each pair. These data were subjected to a Wilcoxon signed-rank test to determine statistical significance. This test was used to determine preference for each of the pairs and for the populations by using the means of the differences in responses for each of the species and pairs. The Wilcoxon test compares the distribution and mean of the response differences to each pair used at that site with regard to the total number of copulations. Data were analyzed to test the null hypothesis that the distribution mean of the differences was equal to 0, using the JMP statistical program.

Results

The data collected in this study show clearly that mate preference by the males of *P. glaucus* and *P. canadensis* is not random, even at great distances from the hybrid zone between the two populations. We have found that the males of both *P. glaucus* and *P. canadensis* prefer the females of *P. glaucus* in these paired tests.

The data gathered from Florida in both 1997 and 1998, and from Michigan in 1997 reveal a clear pattern of mate choice. The null hypothesis stated at the beginning of this study was: That there will be no difference in the behavior of *P. glaucus* and *P. canadensis* males from populations long distances from the hybrid zone towards heterospecific pairs of size matched, tethered females. Both populations chose females of *P. glaucus* for their mate choices, thus rejecting the null hypothesis.

In 1997 *P. glaucus* males in Florida were particularly selective in their choice of mates (Figure 5). Analysis showed that 94.2% of the copulations occurred with *P. glaucus*. 67% of the touches were made towards *P. glaucus* (n=112) and 87.5% of the attempted copulations were with *P. glaucus* (n=56) (Table 1). The total number of female pairs tested in spring of 1997 was 19 (1 set of paired females = 1 data point). A column of data showing the difference between

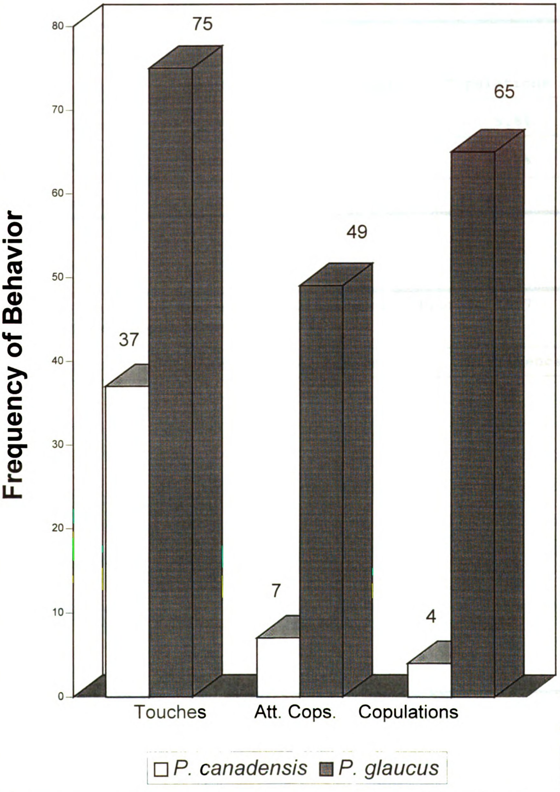


Figure 5: 1997 Florida Totals. Frequncy of behavioral classes exhibited by male *P. glaucus* towards paired females.

the total number of copulations for each female from the 19 pairs was generated (Table 2).

Table 1: March\April 1997 Florida Data Table.

Pairs n = 19	**Touches**	**Attempts**	**Copulations**
P. canadensis:	**33%**	**16.5**	**5.8%**
P. glaucus:	**67%**	**87.5%**	**94.2%**
Total encounters:	**112**	**56**	**69**

Table 2: Copulation Numbers and Differences, Florida 1997.
Species: Total Copulation Numbers

Pairing (Pg/Pc)	***P. glaucus***	***P. canadensis***	**Difference**
Pg1/Pc1	0	0	0
Pg2/Pc2	6	1	5
Pg1/Pc3	5	0	5
Pg3/Pc1	3	0	3
Pg4/Pc4	1	0	1
Pg3/Pc5	8	0	8
Pg1/Pc4	6	1	5
Pg5/Pc1	2	0	2
Pg6/Pc4	1	0	1
Pg7/Pc6	1	0	1
Pg3/Pc3	0	0	0
Pg6/Pc6	0	0	0
Pg7/Pc7	6	0	6
PgGA/PcMI	3	0	3
Pg8/Pc8	5	0	5
Pg6/Pc7	2	0	2
Pg8/Pc7	6	1	5
Pg8/Pc6	5	0	5
Pg7/Pc8	5	1	4

In Florida, 1997, the statistical test used showed that there was indeed a difference in the way that males were reacting to the individual females placed out in heterospecific pairs (Table 3). The mean difference, in number of copulations per pair, from the estimated null of 0.0 was 3.16, with a standard deviation of 2.41. Standard error was 0.55. The signed rank produced for this data was 60.0, P <.0001.

Table 3: Statistical analysis of FL 1997 Difference Data.
Actual Mean = 3.15789
Hypothesized value = 0.0
St. Dev. = 2.40978
St Error = 0.55284
N= 19
Signed Rank (Wilcoxon Test) = 60.000, **P** = 0.000

In March and April of 1998 the Florida populations were again bioassayed. It was found that the pattern seen in 1997 had not changed significantly. The population levels in 1998 were lower than in 1997 (Scriber et al 1998) and consequently not as much data per pair was collected. All of the *P. glaucus* males choose to copulate with the *P. glaucus* females of the pairs (n=22 pairs) (Figure 6). 59% of the touches were with conspecific females (n=29), and 80% of attempted copulations with *P. glaucus* females as well (n=20) (Table 4).

Table 4: March\April 1998 Florida Data Table.

Pairs n = 22	**Touches**	**Attempts**	**Copulations**
P. canadensis:	**41%**	**20%**	**0.0%**
P. glaucus:	**59%**	**80%**	**100%**
Total encounters:	**29**	**20**	**30**

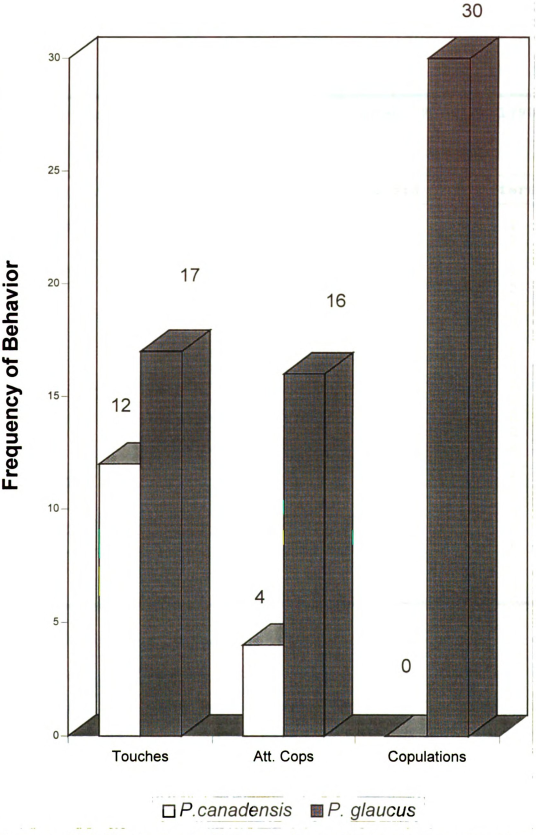


Figure 6: 1998 Florida Totals. Frequncy of behavioral classes exhibited by male *P. glaucus* towards paired females.

The numbers of copulations for each female of each paring was placed into columns and a column of differences was generated (Table 5).

Table 5: Copulation Numbers and Differences. Florida 1998.

Species: Total Copulation Numbers

Pairing (Pg/Pc)	***P. glaucus***	***P. canadensis***	**Difference**
Pg21/Pc14	1	0	1
Pg5/Pc2	3	0	3
Pg4/Pc1	1	0	1
Pg10/Pc5	2	0	2
Pg15/Pc7	2	0	2
Pg5/Pc4	9	0	9
Pg2/Pc2	0	0	0
Pg1/Pc1	1	0	0
Pg8/Pc13	0	0	0
Pg21/Pc9	0	0	0
Pg8/Pc14	0	0	0
Pg15/Pc3	0	0	0
Pg6/Pc1	0	0	0
Pg4/Pc2	0	0	0
Pg1/Pc2	4	0	4
Pg16/Pc2	0	0	0
Pg18/Pc12	1	0	1
Pg12/Pc9	3	0	3
Pg16/Pc7	0	0	0
Pg10/Pc12	0	0	0
Pg18/Pc4	0	0	0
Pg12/Pc5	0	0	0
Pg1/Pc4	2	0	2
Pg13/Pc1	1	0	1

The data collected in 1998 in Florida showed a very significant difference in the in the way that the two females of the pairs were chosen. The number of pairs used in 1998 was 22. Data was gathered in only 12 of these pairs, but the others were included because there were males in the area and they did have the opportunity to react to the females in these pairs. The mean of the differences in copulation total for each of the females in these parings was 1.25 with a standard deviation of 2.03 and a standard error of 0.41. The Wilcoxon test returned a value of 39.0, which correlates to a P value of .0061 (Table 6).

Table 6: Statistical analysis of FL 1998 Difference Data.
Actual Mean = 1.25
Hypothesized value = 0.0
St. Dev. = 2.03
St Error = 0.41
N = 22
Signed Rank (Wilcoxon Test) = 39.0, P = 0.000

The data collected in June of 1997 at sites in Northern Michigan did not support what was predicted (Figure 7). Of 493 total copulations observed in Northern Michigan, 82.3% were with the heterospecific female, *P. glaucus*. Out of 355 touches, 58.4% were for the glaucus females. The totals for attempted copulations showed that 79% of 128 encounters were for *P. glaucus* as well (Pairs=25) (Table 7).

Table 7: 1997 Michigan Data Table.

Pairs n = 25	**Touches**	**Attempts**	**Copulations**
P. canadensis:	**41.6%**	**21%**	**17.7%**
P. glaucus:	**58.4%**	**79%**	**82.3%**
Total encounters:	**355**	**128**	**493**

These data were analyzed as before, using the same criteria and set up (Table 8).

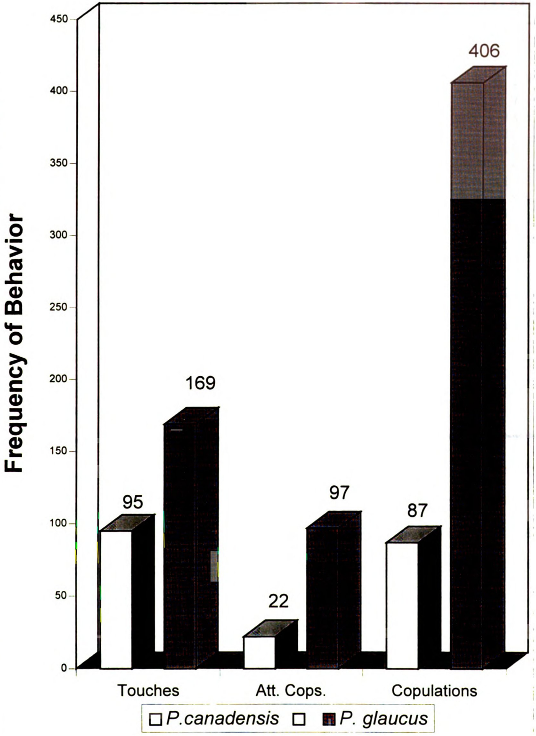


Figure 7: 1997 Michigan Totals. Frequency of behavioral classes exhibited by male *P. glaucus* towards paired females

Table 8: Copulation Numbers and Differences. Michigan 1997.
Species: Total Copulation Numbers

Pairing (Pg/Pc)	***P. glaucus***	***P. canadensis***	**Difference**
Pg1/Pc1	10	0	10
Pg2/Pc2	20	5	15
Pg3/Pc3	29	8	21
Pg4/Pc4	12	0	12
Pg4/Pc1	7	0	7
Pg5/Pc1	24	1	23
Pg5/Pc4	23	1	22
Pg2/Pc7	18	8	10
Pg2/Pc3	8	2	6
Pg3/Pc2	28	8	20
Pg5/Pc6	8	11	-3
Pg6/Pc4	21	5	16
pg8/Pc8	33	4	29
Pg7/Pc6	22	5	17
Pg7/Pc8	2	0	2
pg8/Pc6	4	1	3
Pg2/Pc9	30	3	27
Pg10/Pc10	16	9	7
Pg2/Pc10	13	2	11
Pg10/Pc9	7	0	7
Pg7/Pc1	5	2	3
Pg8/Pc3	21	6	15
Pg6/Pc8	20	5	15
Pg11/Pc11	2	1	1
Pg8/pc4	6	0	6

The data gathered in Michigan in 1997 was analyzed using the same test. The column of differences produced gave a mean difference of 12.32, with standard deviation of 8.08, and standard error of 1.62. There were 25 pairs of females used for data collection in Michigan. The Wilcoxon test returned a signed rank that was 162.5, giving a P value of 0.000 (See Table 9).

Table 9: Statistical analysis of MI 1997 Difference Data.

Actual Mean = 12.32
Hypothesized value = 0.0
St. Dev. = 8.08
St Error = 0.1.62
N = 25
Signed Rank (Wilcoxon Test) = 162.5, P = 0.000

Discussion

The data collected in this study shows clearly that mate preference by the males of *P. glaucus* and *P. canadensis* is not a random phenomenon, even at great distances from the hybrid zone between the two populations. We have found that the males of both *Papilio glaucus* and *P. canadensis* prefer the females of *P. glaucus* in these paired tests.

The 1997 experiments in Florida showed that 94.2% of *P. glaucus* males chose to mate with the conspecific female of the pairs (n=19). We observed a similar pattern in spring of 1998 even though the population levels were lower than in 1997. *P. glaucus* males choose to copulate with the *P. glaucus* females of the pairs we placed out in all cases. We tethered a total of 22 pairs in spring of 1998 but recorded data in the form of copulations on only 12 of those pairs.

Results from Florida suggest that *P. glaucus* males are able to differentiate between females of *P. glaucus* and *P. canadensis*, and that they prefer to mate with the conspecific female. This result was not totally unexpected as species specific mate cues are often an important factor in mate selection.

The data collected in June of 1997 showed that males in Michigan were as selective as those observed in Florida. Out of 493 total copulations involving free flying *P. canadensis* males in Northern Michigan, 82.3% were with the heterospecific female, *P. glaucus* (Pairs=25).

It can be stated with confidence that the males counted in the Florida data were not counted twice since *P. glaucus* in the area were being marked for a population study (Scriber et al. 1998 submitted). Previously unmarked males that copulated were removed from copulation and were then marked and recorded. None of the males returned twice. This not only provided proof that the same males were not being counted twice but also allowed for a record of the sizes and wear class (approximate age) of each of the males. This was not so in Michigan as *P. canadensis* males that copulated in Michigan were not being marked. It was noted that all but a few males left the area very rapidly once they were separated by hand from copulation with the female. However, the number of copulations by males recorded in Northern Michigan, as well as the number of pairs used, was large enough that it would likely outweigh any possible pseudo-replication caused by returning males.

This study has shown that *P. canadensis* males have a strong preference for the heterospecific female of these pairs, and that species conspecific mate cues were not preferred here. This allows for rejection of the null hypothesis. Obviously these results are in conflict with the prediction of no clear preference for females by males in either population long distances from the hybrid zone. It can be surmised that since the two species likely arose from an allopatric speciation event during the last ice age that there may be some ancestral trait in females of *P. glaucus* that is more attractive to the males of *P. canadensis* than their own females. This idea, of ancestral attractiveness, is not unprecedented. Other researchers have used this idea to explain some behaviors that they found in their systems, including ovipositional preference in *P. canadensis* females for a toxic (for them) host; *Liriodendron tulipifera* (Bossart & Scriber 1995), mating ornamentation systems in least auklets (Jones and Hunter 1998) and female mate choice in some *Shizocoza* wolfspiders (McClintock & Uetz 1996).

Interestingly, the study by Bossart & Scriber (1995) showed that some females from a population of *P. canadensis* in Alaska had a strong preference for using tulip tree as an ovipositional host, especially since they are thousands of miles from any tulip tree and they are toxic to the larvae. Those females with lesser preference for tulip tree were collected from near the plant tension zone, where they could have been undergoing selection against using the toxic tulip tree. So thus, it appears that the species can exhibit behavior that is non-adaptive. It is quite possible that tethering within the hybrid zone itself will reveal that those *P. canadensis* males might be more likely to select conspecific females bearing in mind that there may be evolutionary pressures (e.g. via this reinforcement of prezygotic isolating mechanisms) to be more accurate in their selections.

The results found in this study help to confirm that the males of the *Papilio glaucus* species group are characterized by strong visual acuity, as is commonly found in many species where the males engage in puddling behavior(Arms et al. 1974). With male mate locating behavior associated with patrolling (Lederhouse 1995) this is also expected. The males of *P. canadensis* and *P. glaucus* patrol roadsides, forest edges, steam edges, nectar source areas and hedgerows (personal observation). The Western species *P. rutulus, P. eurymedon* and *P. multicaudatus* also patrol using the canyons and hilltops as the areas that they frequent (Brower 1959, Shields 1968, Scott 1975, 1983). There are other patrolling Papilionidae that have been documented in other countries as well (Yamashita 1995).

Females in the *P. glaucus* group are known to mate multiple times from studies counting spermatophores in some species (Lederhouse et al. 1989, Lederhouse 1995), but this is not the case in all species of Papilionidae. There are many species that have evolved complex arrangements of mating plugs designed to mechanically ensure that the female does not re-mate (Orr 1995, Matsumoto & Suzuki 1995). This investment of resources by the male ensures that no other male fertilizes the eggs of that particular female. This is important to the male that mates with the female first because if the female is able to mate again the new spermatophore would likely displace his sperm through sperm precedence (Parker 1970).

Conversely, the investment made by males of the *P. glaucus* group is in the spermatophore itself. The spermatophore that these butterflies pass to the female can often contain 5% or more of their total body weight (Rutowski et al. 1984). This nuptial gift also often contains sugars, proteins and other micronutrients that the female may be able to incorporate and use in somatic maintenance and egg production (Boggs & Gilbert 1979, Boggs 1981, 1990).

Generally speaking, the larger the spermatophore that is passed to the female, the longer the time it will be until she mates again. This helps to ensure that more eggs will be fertilized using the sperm provided by the male producing that spermatophore (Lederhouse et al. 1989).

Some other factors that may be considered as possible reasons that *P. canadensis* males choose heterospecific mates include; (1) the amount of blue present on the hindwings of most of the *P. glaucus* females. The blue is much more extensive than that on the hindwings of *P. canadensis* females and may be acting as a mate attractant. (2) the possibility that males are choosing the females of *P. glaucus* simply because they are novel to them, (3) That there is a certain amount of hybrid vigor present in the F1 crosses of these species (Scriber & Deering pers. obs.). Hybrid vigor is a well known phenomena (Collins 1984) and may manifest as increased survivorship due in part to faster developmental rates. This may account for the large proportions of hybrids in some areas such as *Hyalophora* hybrids in California (Collins 1984). Other evidence of hybrid vigor that often appear are increased disease resistance (Scriber & Deering pers. obs.) or the fact that hybrids between *P. glaucus* and *P. canadensis* are able to utilize the host ranges of both populations as they can detoxify both the hosts of *P. canadensis,* mainly *Populus sp.,* and those of *P. glaucus*, *Liriodendron tulipifera,* and *Magnolia virginiana* (Scriber et al. 1995). Finally, (4) given that the females are multiple maters (Drummond 1984) they do not have to be selective, as their mate choices may have little overall effect as far as fertilization goes. The females of both *P. glaucus* and *P. canadensis* are known to be multiple maters (Lederhouse & Scriber 1987, 1989). Other researchers have looked at the role of sperm precedence and cryptic sexual selection in this and other mating systems (Eberhardt 1996), and are currently looking into the possibility that cryptic sexual selection by females may override sperm precedence (Stump & Scriber, unpublished). If a female can choose to use a spermatophore from a conspecific male even though she may have a mixture of spermatophores in her bursa, then male mate choice becomes less critical in terms of reinforcement.

If reinforcement is taking place between these two species it must be occurring only in the narrow confines of the hybrid zone or very near to it. We were not able to test for this reinforcement due to lack of suitable field sites in and around the hybrid zone in Michigan and this is an area that needs to be more closely scrutinized in the future. A new way that researchers may be able to tell if males of *P. canadensis* and *P. glaucus* are choosing heterospecific or conspecific mates in the hybrid zone is by looking at individual specimens collected from these areas. By running the specimens through an electrophoretic analysis designed to identify diagnostic allozyme markers, or by using diagnostic larval traits (Scriber 1998) it may be determined if the individual was a hybrid or not. Additionally, maternally inherited mitochondrial DNA from the specimen would reveal the direction of the hybrid cross and gene flow. Since there are differences in the mitochondrial DNA from these two species, and since this DNA is always passed maternally we can tell if the mother of an individual was of *P. glaucus* or *P. canadensis* lineage.

The data that has been recorded so far disproves the null hypothesis that mate choice by male *P. glaucus* and *P. canadensis* would be an effective form of prezygotic reproductive isolation by itself. If there were no other barriers to gene flow involved in this system the hybrid zone between the two species would be spreading Southward as males of both species chose *P. glaucus* females for mates. In effect, there would facilitated introgression to the South as males of both *P. glaucus* and *P. canadensis* will choose *P. glaucus* females over *P. canadensis* females to mate with. Since the hybrid zone appears to have been stable for at least a 100 years (Edwards 1884), corresponding closely with the Boreal/Temperate plant transition zone and with Rothschild and Jordans (1906) and Remingtons' observations (1968), it may be more likely that other prezygotic, postzygotic or ecological factors are constraining the movement of these *Papilio* species across the hybrid zone.

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