THE UNIVERSITY OF CHICAGO

MATE CHOICE BEHAVIOUR AND THE EVOLUTION OF BUTTERFLY SPECIES

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES
AND THE PRITZKER SCHOOL OF MEDICINE
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

COMMITTEE ON EVOLUTIONARY BIOLOGY

BY
LAURA CATHERINE SOUTHCOTT

CHICAGO, ILLINOIS
JUNE 2019

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

First I have to acknowledge that the six years of my PhD have been horrible, but that I'm fine now thanks to medication, therapy, and support from my friends. I want to thank everyone who is working towards improving the mental health of graduate students through both immediate interventions and cultural change. Your task seems Sisyphean, but it's absolutely necessary, and academia is slowly becoming better and more inclusive because of your efforts.

I owe a great deal to the Smithsonian Tropical Research Institute, where the work for chapters 3 and 4 was conducted. Thanks especially to Owen McMillan for allowing me to use the insectary facility and for advice, encouragement, and barbecueing. I am forever indebted to my capable and enthusiastic field assistants: Emma Tomaszewski, Aaron Goodman, Holly Black, Amber Antonison, Lina Melo, Diego Vergara, and Chantelle Doyle. Without them, I'd have far fewer data points and and even fewer sanity points. I am deeply grateful as well to Oscar Paneso and Liz Evans for their incredible butterfly rearing expertise and problem solving skills. I also want to thank the rest of the Gamboa community, especially Kathy Darragh, Kelsey Byers, Sylvia Garza, and again Liz Evans, who saw me through some of my worst months and did the best they could for me.

I thank my labmates and friends at the University of Chicago and elsewhere, who helped with research logistics, experimental design, stimulating scientific debate, baked goods, friendship, and understanding. They are, in particular order and likely with some omissions (for which I beg forgiveness), Sara Jackrel, Allison Johnson, Misha Blizard, Natalia Piland, Katherine Silliman, Roberto Márquez, Dave Grossnickle, Erica Westerman, Darcy Ross, Nick VanKuren, Darli Massardo, Emelyne Gaudichau, Benjamin Blanchard, Jordan Greer, Abhimanyu Lele, Ayse Tenger-Trolander, Daniela Palmer, Sumitha Nallu, Tim Thurman, Crystal Maier, Rich Merrill, Valerie McMillan, and Supriya (who was in it with me from the start).

The staff of the Committee on Evolutionary Biology and the Department of Ecology and Evolution were vital to my time in graduate school, helping navigate bureaurocracy, facilitate reimbursements, and occasionally offer loans in times of need. Thanks especially to Carolyn Johnson, Libby Eakin, and Bonnie Brown. I am also grateful to my therapist, Aileen Schloerb, without whose help

my PhD would have gone a lot worse.

Finally, my family has always believed in me and continues to think I'm smarter than I am: thank you for putting up with me. And thank you, above all, to Alejandro Sanchez, who has carried me through the worst of times with a big goofy grin.

ABSTRACT

Species are the fundamental unit of biodiversity, and the evolution of new species is a fruitful area of research. In sexually reproducing organisms, reproductive isolation is considered fundamental to speciation, and the evolution of mate choice is thus an important mechanism of species origin and maintenance. In this dissertation, I focus on the butterfly genus *Heliconius* to elucidate how mate choice has contributed to speciation. I first simulated genomic divergence in different demographic and selective scenarios to understand whether a nonlinear increase in the highly differentiated part of the genome over time denotes the actions of selection and gene flow during speciation. This pattern, first noted among species of *Heliconius*, arises in a wide range of scenarios, including neutral divergence with no gene flow, while high gene flow and selection disrupt it. Next I tested mate preference of live butterflies of two species, *H. cydno* and *H. pachinus*, that regularly exchange genes. I found a preference for conspecific mates in both sexes, and thus that both sexes contribute to premating reproductive isolation. This is one of the first demonstrations of interspecific female choice in *Heliconius*. I further examined hybrid males' preference for both parental species and found that, contrary to theoretical predictions, preference was not correlated with two wing colour pattern elements thought to play a role in mate choice. Finally, I conducted a meta-analysis of premating isolation among *Heliconius* species and subspecies, finding small effects of both genetic differentiation and experimental design. The long history of research on genomics and behaviour of *Heliconius* butterflies continues to shed light on the role of mate choice in speciation and reveal directions for future research.

CHAPTER 1

INTRODUCTION

All eukaryotic life is related, yet genotypes and phenotypes are not continuously distributed. Rather, organisms tend to form clusters that seldom exchange genes and which we recognize as species: arguably the only real taxonomic unit. Naturally, how species evolve is a productive area of research, and one that caught my interest early in my scientific career. For a young evolutionary biologist who also insisted on minoring in Latin, a genus of butterflies famous for speciation research and named for the Muses and other figures of classical mythology was irresistable.

Heliconius butterflies have become useful models of speciation and hybridization, as they are amenable to captive rearing and crossing, have a short generation time, differ in easily distinguishable wing colour patterns largely controlled by Mendelian loci, and naturally hybridize in certain cases. This research program has uncovered extensive adaptive introgression within the genus and found evidence for "magic traits" causing the evolution of reproductive isolation (Merrill et al. 2015). I explore *Heliconius* speciation and mating behaviour from three perspectives in this dissertation: simulations of genomic divergence, behavioural assays of interspecific mate preference, and a meta-analysis of premating reproductive isolation.

In my first data chapter, I tested the hypothesis that a combination of divergent natural selection and gene flow could produce a signature in which the proportion of the genome that lies in highly divergent outlier regions increases faster than linearly over time (Kronforst et al. 2013). Using coalescent simulation, I found that this pattern arises in many scenarios with or without gene flow and with or without selection. It only breaks down when gene flow is exceptionally high—possibly too high for speciation to proceed in nature. This finding should inform future comparative genomic studies in *Heliconius* butterflies and beyond.

In Chapter 3 I investigated an understudied aspect of *Heliconius* speciation: female mate choice. Most studies of mate choice in these butterflies have focused on males, because they usually initiate courtship and are therefore easier to test. However, both theoretical considerations and preliminary results suggested that female choice is an important part of premating isolation. Females may pay a

higher cost when they hybridize because they remate less often than males (Walters et al. 2012) and so a greater proportion of their offspring would be maladapted hybrids. Further, mate choice trials that allowed both male and female choice had higher measured reproductive isolation than those that only permitted male choice (Mérot et al. 2017). In this chapter I showed that *H. cydno* females prefer to mate with conspecific males over males of the sister species *H. pachinus*.

I examined mate choice behaviour in more detail and turned to male choice for Chapter 4. I confirmed that *H. pachinus* and *H. cydno* males preferentially court conspecific females and investigated the mate preferences of hybrid males. F2 hybrid preference was of particular interest because it could be used to test hypotheses about the genetic architecture of speciation. Divergence with gene flow can be facilitated when the genes underlying mate preference are genetically correlated with the genes underlying divergent traits. In *Heliconius*, wing colour patterns are under divergent natural selection and are also mate choice cues. I tested whether the two traits co-segregate in F2 hybrids, as has been shown in other congeners (Kronforst et al. 2006b; Merrill et al. 2011b, 2019). Surprisingly, I found no such correlation, suggesting that there may be standing genetic variation within species for mate preference.

Across *Heliconius*, courtship and mating of heterospecifics occurs frequently in experimental settings. For the final part of Chapter 4 I gathered data from published studies on interspecific mate choice in *Heliconius* to examine factors that predict how often hybridization occurs—factors that included properties of both the species pair and the experimental design. This meta-analysis sheds light on what *Heliconius* can tell us about premating isolation, as well as what researchers should consider when embarking on butterfly mate choice experiments.

CHAPTER 2

A NEUTRAL VIEW OF THE EVOLVING GENOMIC ARCHITECTURE OF SPECIATION

This chapter was first published as "Southcott, L. and M. R. Kronforst, 2017. A neutral view of the evolving genomic architecture of speciation. Ecology and Evolution, 7:6358-6366" and is reproduced here with permission. Supplemental material referred to in this chapter is included as Appendix A, "Supplemental material for Chapter 2".

Introduction

PECIATION is responsible for the diversity of life on earth, and understanding its mechanisms is of great interest to evolutionary biologists. A genetic approach to this field seeks to identify loci driving speciation and characterize the changing patterns of divergence across the genome during speciation. Technological progress in genetics and genomics is rapidly advancing this research program (Twyford and Ennos 2012; Seehausen et al. 2014). Recently, the availability of whole genome sequences and reduced representation genomic data in an increasing variety of species has begun to make tests of predictions concerning genome-wide patterns of divergence possible (Roesti et al. 2012; Renaut et al. 2013; Gagnaire et al. 2013; Soria-Carrasco et al. 2014).

An increasingly accepted view is that genomic divergence between closely related species is often heterogeneous (Wu 2001; Rieseberg and Burke 2001; Nosil et al. 2009; Feder et al. 2012). Certain genomic regions, namely those tightly linked to loci causing reproductive isolation between occasionally hybridizing taxa, may be especially resistant to gene flow, while unlinked neutral differences easily introgress between species. When selection is strong and populations are small, the region linked to the selected locus can increase in size via divergence hitchhiking (Via and West 2008; Feder and Nosil 2010). At the same time, strong reproductive isolation promotes divergence across the whole genome

(Nosil and Feder 2012); during ecological speciation, the loci underlying reproductive isolation experience divergent natural selection (Schluter 2009).

Methods to identify regions of the genome that contain targets of divergent selection between species make use of the population genetic prediction that loci subject to positive selection will exhibit low within-population variance but high between-population variance when compared to loci that have not undergone the same selection regime. Thus, genome scans in which a metric of population differentiation such as F_{ST} is calculated for consecutive sequence windows or for markers dispersed across the genome can be used to identify areas with unusally high differentiation (speciation islands), areas which should be enriched for genes causing reproductive isolation between taxa (Turner et al. 2005). Such methods have limits, and may be unsuitable when used alone to infer differential gene flow among genomic regions (Cruickshank and Hahn 2014; Burri et al. 2015; Wolf and Ellegren 2016). Indeed, a suite of processes other than divergent selection on two alternative alleles can create peaks in the F_{ST} landscape (Excoffier and Ray 2008; Bierne 2010; Roesti et al. 2014), and the power to detect outliers may vary across the genome (Cruickshank and Hahn 2014). However, outlier scans provide useful guides for identifying loci involved in species differences and thus have become a standard tool in speciation genomics (Beaumont 2005; Strasburg et al. 2012; Wolf and Ellegren 2016).

While outlier analyses were initially used to compare two populations or species, a new goal is to compare outlier scans among multiple pairs of species to investigate parallel adaptation/speciation and divergence at different stages and in different demographic situations. For example, four independently evolved pairs of host-plant races of the stick insect *Timema cristinae* exhibited parallel divergence in some regions of the genome, but other highly differentiated regions were unique to each population pair (Soria-Carrasco et al. 2014). All *T. cristinae* host-race pairs in Soria-Carrasco et al. (2014) were young and had high gene flow, but areas of high divergence, as determined by a hidden Markov model, comprised 8-30% of the genome, with the largest percentage found in the one pair that was geographically separated rather than adjacent.

Other studies have begun to compare genome-wide differentiation patterns across stages of divergence. In lake and stream threespine stickleback (*Gasterosteus aculeatus*), the median genome-wide

F_{ST} was higher in pairs with greater morphological differences (Roesti et al. 2012). Independently evolved pairs of dwarf and normal lake whitefish (*Coregonus clupeaformis*) also had higher overall F_{ST} when they were more phenotypically divergent (Gagnaire et al. 2013), and increasingly genetically differentiated population pairs had more large divergent regions (Renaut et al. 2012). Highly divergent regions comprised a smaller portion of the genome in comparisons of sunflower ecotypes than in comparisons between species (Andrew and Rieseberg 2013). Comparisons among several sunflower species, however, found that the size and number of divergent regions differed little among pairs in different geographic (and gene flow) contexts (Renaut et al. 2013). On the other hand, when three phylogenetically independent species pairs were compared, mean SNP F_{ST} was larger in older species pairs (Renaut et al. 2014).

Currently, there is relatively little theoretical work that explores expected genome-wide patterns during divergence with gene flow. Flaxman et al. (2014) found that, for certain combinations of migration rate and strength of selection, the effective migration rate (a measure of reproductive isolation) decreases gradually at first, then sharply. This rate change coincided with an abrupt change in the rate of increase of divergently selected loci. Thus, during speciation with gene flow the genome may transition from a porous phase characterized by free gene flow of alleles that are not under divergent selection to a phase dominated by reproductive isolation across the whole genome and widespread linkage disequilibrium. This process has been termed "genome-wide congealing" (Flaxman et al. 2014; Feder et al. 2014). The genome-wide congealing hypothesis joins other theoretical predictions of nonlinear dynamics during speciation with gene flow. Adaptive dynamics suggests that speciation can occur abruptly due to disruptive selection at certain points in bivariate trait space (Geritz et al. 1998; Ito and Dieckmann 2012). Coupling of incompatibility loci, under certain conditions, feeds back to cause the evolution of further coupling, leading to nonlinear transitions across hybrid zones and over evolutionary time (Barton 1983; Barton and Bengtsson 1986; Barton and De Cara 2009; Bierne et al. 2011). However, nonlinearity even without selection and gene flow may occur. The snowball theory, for instance, predicts a faster than linear increase in the number of genetic incompatibilities as a result of epistastic interactions among linearly increasing substitutions (Orr 1995). Outlier scans

examine the distribution of these linearly increasing substitutions without considering their effects (including epistatic effects). Under genome-wide congealing, nonlinear increases in outliers might be expected. Thus, the behaviour of allopatrically diverging populations in the absence of selection should be considered for comparison with the predictions of genome-wide congealing models.

To quantify how genome-wide divergence patterns in nature change with time since speciation, Kronforst et al. (2013) examined divergence between three species of *Heliconius* butterflies from Costa Rica. They found that the proportion of the genome that lay in highly divergent regions increased faster than linearly with increasing time since divergence between each pair of species. They attributed this result to gradually attenuating gene flow during speciation, with a tipping point hybridization rate above which divergence is inhibited and below which it accelerates—a suggestion similar to a genomic "congealing" process. However, Flaxman et al. (2014) predicted speciation in *Heliconius* to proceed without such a nonlinear transition, because it often involves few genes of large effect (Nadeau et al. 2012; Kronforst and Papa 2015).

To explore whether this faster than linear increase could be produced by processes other than selection interacting with gene flow, we simulated neutral evolution in allopatry and compared it to various scenarios of speciation with gene flow and selection and to Kronforst et al.'s (2013) results for *Heliconius*. Our results suggest next steps in examining genome-wide patterns of divergence and highlight the need for null model comparisons in the emerging field of speciation genomics.

Methods

Simulations

We generated gene trees using the neutral coalescent modelling software ms (Hudson 2002), then evolved sequences along these trees with Seq-Gen (Rambaut and Grassly 1997) under a Jukes-Cantor model. We simulated three scenarios: no gene flow, gene flow between sister species for the first 2N generations following divergence ("early gene flow"), and gene flow for the most recent 2N generations ("recent gene flow"), where N is the effective population size. These scenarios correspond to

allopatric speciation, speciation with gene flow followed by complete reproductive isolation, and secondary contact following allopatric divergence respectively. We simulated each scenario 30 times, and each time simulated ten sequences from each of 16 species comprising eight sister pairs of varying ages (from 2N to 16N generations; Figure 2.1). Each species had the same constant effective population size (N); we simulated N = 10^6, 10^5, and 10^4. Each sequence was 100 kbp long with a recombination rate of 10^{-8} per site per generation and a mutation rate of 5×10^{-9} per site per generation. Because of the short genome length, we did not attempt to vary recombination or mutation rate within the genome, nor could we investigate long-range linkage disequilibrium. For N = 10^6, we simulated both unidirectional and bidirectional gene flow with migration parameters of 4Nm = 10, 1, 0.1, 0.01, and 0.001. For N = 10^5, we simulated unidirectional migration of 4Nm = 0.0001, 0.01, and 1; and for N = 10^4, 4Nm = 0.00001, 0.001, and 0.1. These correspond to the same migration rates (m) as simulations of N = 10^6 and 4Nm = 0.001, 0.1, and 10.

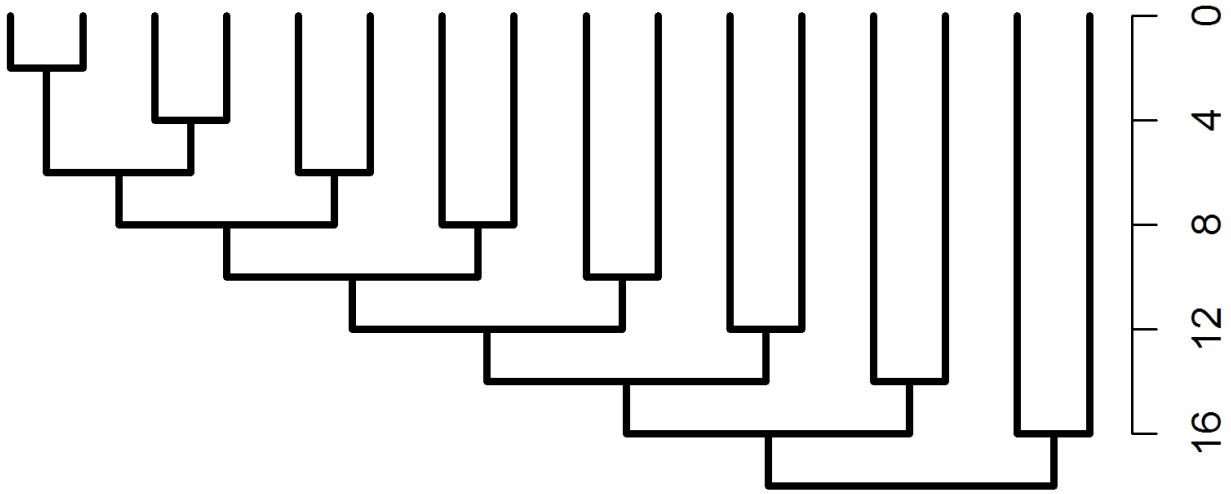

Figure 2.1: Relationships among species simulated in this study. F_{ST} was only calculated between sister species to avoid phylogenetic nonindependence. Scale is in units of N generations, where N is the effective population size of 10^6.

For a subset of these demographic scenarios (N = 10^6 and no gene flow, unidirectional early gene flow with 4Nm = 10, and unidirectional recent gene flow with 4Nm = 0.001, 0.01, 0.1, 1.0, and 10.0), we also investigated gene trees for regions adjacent to a selected locus using msms (Ewing and Hermisson

2010), a coalescent simulator based on ms that incorporates forward-in-time simulations of selection on single loci. We modelled gene trees for 100kb adjacent to a selected locus with two alleles under divergent selection between species (s = 0.01 or 0 for homozygotes and s = 0.005 for heterozygotes). As for the neutral simulations, we generated sequences with Seq-Gen. The full ms/msms and Seq-Gen input parameters are presented in the supplemental material.

Analysis

We used the package PopGenome (Pfeifer et al. 2014) in R (R Core Team 2013) version 2.15.0 to calculate F_{ST} using polymorphic loci (Hudson et al. 1992) between sister species for each nonoverlapping 500 bp window in the simulated genomes. For each run of the simulation (160 sequences from eight species pairs), all F_{ST} values were pooled to set a common threshold for identifying outliers across species. We considered the 80th, 95th, and 99th percentiles as thresholds above which an F_{ST} value was considered an outlier. We also applied a relaxed threshold method in which, if all intervening windows between two consecutive 95% outlier windows had F_{ST} larger than the 75th percentile, these windows were also designated outliers (Kronforst et al. 2013). We also calculated average between-population nucleotide divergence (d_{xy}, Nei 1987) and number of fixed differences for each species pair.

For each simulation run, we performed linear and exponential regressions, both forced through the origin, of divergent window number versus time since divergence of the species pair. We compared AIC values for the two regressions. Since all scenarios except recent gene flow with a migration rate of 1 or 10 clearly followed an exponential rather than linear curve, we compared the coefficients of the exponential regression (b in $y = a(1 - e^{bx})$) among gene flow scenarios with an ANOVA.

Results

No selection

Results for unidirectional gene flow and $N = 10^6$ are presented here; scenarios with bidirectional gene flow did not differ substantially for the input parameters we tested and are presented in the supplemental material (Figure A1). The number of divergent windows increased faster than linearly when no gene flow or early gene flow up to 4Nm = 10 occurred (Figure 2.2), and an exponential function fit the data better than a linear regression in each simulation (ΔAIC = 7.36 - 59.21). The faster than linear increase was found regardless of the F_{ST} outlier threshold (Figure A2). The rest of the results presented use a 95% + 75% F_{ST} threshold, as described in the Methods.

In the recent gene flow scenario, exponential curves fit the data better than linear functions for migration parameters of 0.001, 0.01, and 0.1 (ΔAIC = 3.35–49.61). For 4Nm = 10, nonlinear regressions did not converge after 1000 iterations for any of the simulated datasets. For 4Nm = 1, nonlinear regression converged in only 10 of 29 simulated datasets.

In all scenarios, d_{xy} increased linearly, more rapidly when there was less migration (Figure 2.3). The number of fixed differences between species pairs increased linearly with no or early gene flow, but began to plateau when higher recent gene flow was considered (Figure A3). Both global and mean per window F_{ST} increased at a decelerating rate in the no gene flow scenario, as expected from coalescent theory (Supplemental Results - Appendix A), and stayed approximately constant over increasing divergence time in the 4Nm = 10 recent gene flow scenario (Figures A4, A5, A6, A7, A8).

Smaller population sizes and correspondingly shorter divergence times differed strikingly from simulations with $N = 10^6$. When $N = 10^4$, the divergent genome grew linearly (Figure 2.4), perhaps because divergence was more rapid than in larger populations and F_{ST} approached 1 closely by the oldest time since divergence. The highest recent gene flow level (4Nm = 0.1) did not completely homogenize the diverging populations. At $N = 10^5$, divergent genome size increased nonlinearly, but less steeply than in simulations of $N = 10^6$ (Figure 2.4). At these lower population sizes, there was again little difference between no migration, early migration, and limited recent migration scenarios.

Divergent selection

Our simulations with a single divergently selected locus produced a nonlinear increase when there was no gene flow or early gene flow (4Nm = 10). For both scenarios, exponential functions fit the data better than linear regression (ΔAIC = 5.34–50.49). This was also the case for scenarios with selection and recent gene flow up to 4Nm = 0.1 (ΔAIC = 2.76–49.55; Figure 2.2). However, selection with recent gene flow of 4Nm = 1 or 10 resulted in a gradual increase in divergent genome size that appeared to plateau, as in a logistic function. For 4Nm = 10, nonlinear regressions did not converge in any simulated datasets, and for 4Nm = 1 they converged in only 12 of 21 datasets.

The number of fixed differences and d_{xy} increased linearly for all scenarios except recent gene flow with 4Nm = 1 or 10. In these scenarios, both statistics underwent a rapid increase between 4×10^6 and 8×10^6 generations followed by a more gradual increase or, for 4Nm = 10, an apparent plateau (Figures 2.3, A3).

When exponential curves were fit to each scenario (both with and without selection, and excluding those with recent gene flow of 4Nm = 10 or 1 due to nonconvergence), the rate of increase of divergent windows versus time (the coefficient of divergence time in the exponential equation) differed among scenarios (ANOVA, F = 3.89, p = 6.1×10^{-6}). However, based on Tukey's HSD test, no scenario differed from the no gene flow, no selection simulations (mean = 0.38, sd = 0.08); only early gene flow of 4Nm = 0.1 without selection (mean = 0.45, sd = 0.09) and recent gene flow of 4Nm = 0.1 without selection (mean = 0.32, sd = 0.09) significantly differed from each other (Figure 2.5).

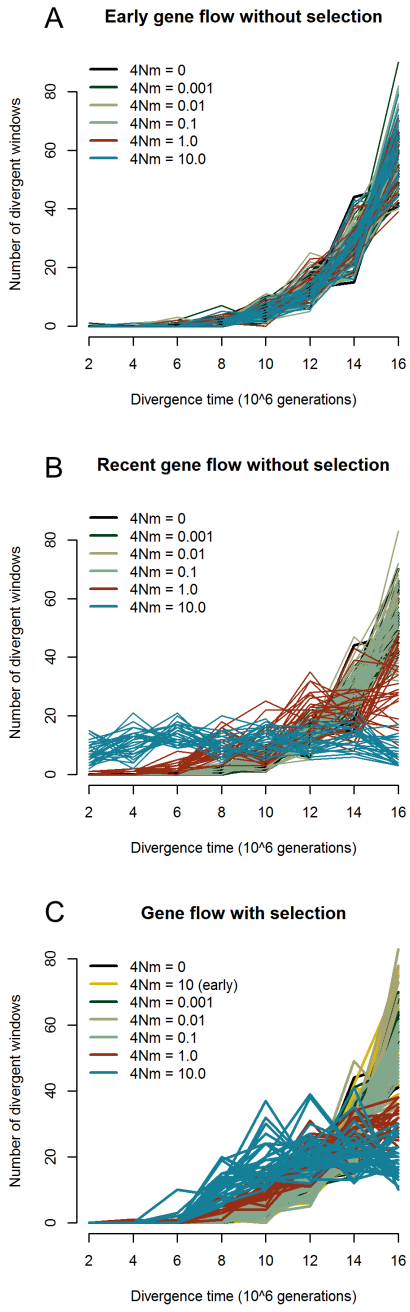

Figure 2.2: A: Divergent genome size (number of outlier windows) increases faster than linearly with divergence time when no selection and no gene flow or various levels of unidirectional early gene flow occurs. B: The highest levels of unidirectional recent gene flow homogenize the genome and prevent this increase. C: The same pattern occurs in simulations with divergent selection and no gene flow, high gene flow, or recent gene flow up to 4Nm = 0.1. Higher gene flow (4Nm = 1.0 or 10.0) and divergent selection result in a nonlinear increase to a plateau. Each line represents a single simulation of eight between-species comparisons.

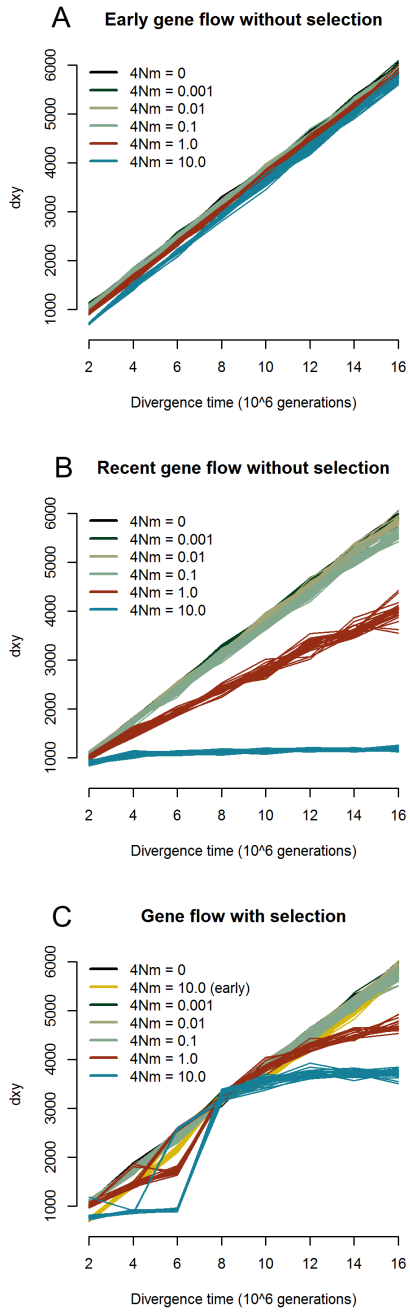

Figure 2.3: A: For simulations without selection, average pairwise substitutions (d_{xy}) increase linearly with divergence time with early gene flow. B: A less rapid increase occurs with higher recent gene flow. C: With selection, d_{xy} increases linearly when gene flow is absent, early, or minimal and recent, but increases nonlinearly with higher recent gene flow.

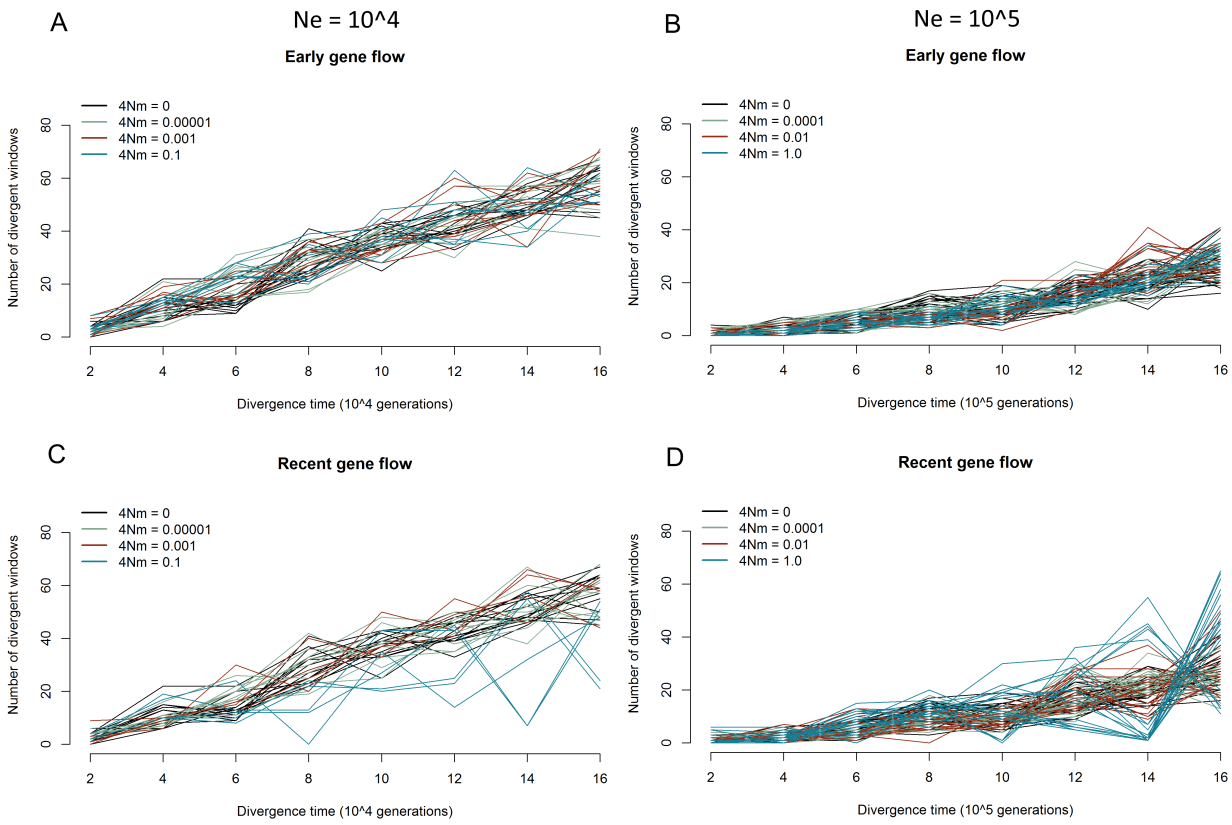

Figure 2.4: Patterns of change in divergent genome size over time with smaller effective population sizes. A: N = 10^4, early migration (for the first 2N generations after divergence). B: N = 10^5, early migration. C: N = 10^4, recent migration (for the most recent 2N generations).D: N = 10^5, recent migration. Note that the x axes are scaled to population size, and thus differ by an order of magnitude between A/C and B/D.

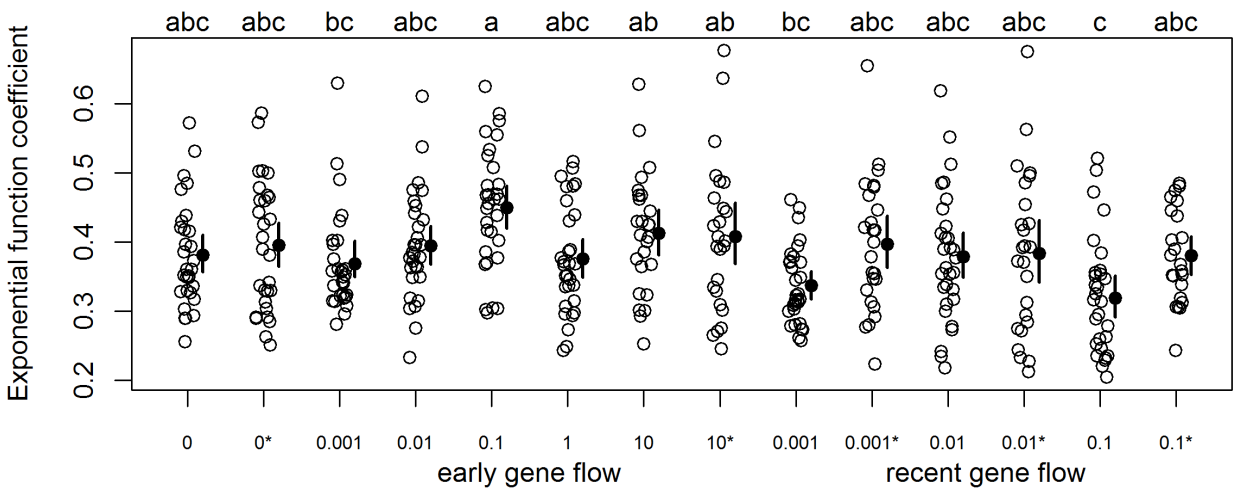

Figure 2.5: The rate of increase in divergent genome size (the coefficient in the exponential function) depends on the extent of recent gene flow. Migration parameter (4Nm) is on the x axis; values with asterisks represent simulations with selection. Open circles are individual data points; filled circles are means with bias corrected bootstrap 95% confidence intervals. Scenarios that do not share the same letter (top) are significantly different based on Tukey's honestly significant difference test.

Discussion

Our results show that, in many demographic scenarios, an exponential increase in divergent genome size provides no evidence for or against gene flow and selection during speciation. When gene flow is extensive and recent, however, divergent selection produces a distinct pattern in both the growth of outlier regions and the increase in d_{xy}, and thus in situations with known gene flow these statistics could provide insight into the action of selection. Likewise, our simulations of smaller populations with shorter divergence times suggest that some neutral scenarios produce linear increases, and thus a nonlinear increase with these demographics might be a useful signature of speciation with gene flow. Below we discuss the implications of our findings for comparative studies of genome-wide divergence and the strengths and weaknesses of this method of outlier analysis.

The *Heliconius* species that inspired this study showed a nonlinear increase in the divergent genome and in the number of fixed differences between species but linear growth of d_{xy} (Kronforst et al. 2013). These results were interpreted as evidence for an interplay between selection and gene flow during speciation, but we did not find this specific combination of patterns in the scenarios we simulated. Our results show that exponential growth of the divergent genome occurs even without selection and gene flow while d_{xy} and the number of fixed differences mirror one another and only show non-linear behavior with selection and gene flow. The disconnect may stem from the fact that *Heliconius* does not closely match the simulation parameters—population size estimates range from 250,000 to 1.8 million across the three *Heliconius* species and divergence times go back an estimated 6 million generations (Kronforst et al. 2013), which is an early time point for the $N = 10^6$ simulations but extremely old (off the charts) for the $N = 10^5$ simulations. However, ample evidence exists that *Heliconius* species are under divergent natural selection, especially on wing colour pattern, and that hybridization occurs among taxa (Bull et al. 2006; Kronforst et al. 2006a; Mallet et al. 2007; Kronforst 2008; Wu et al. 2010; Heliconius Genome Consortium 2012; Martin et al. 2013; Nadeau et al. 2013; Zhang et al. 2016). Furthermore, Kronforst et al. (2013) estimated migration parameters (4Nm) ranging from approximately 1 to well over 10. We might conclude in this case that while the exponential growth of the divergent genome is not in itself informative, the behavior of the number of fixed differences in

Heliconius does support the involvement of selection and gene flow during speciation.

The nonlinear increase in divergent genome size in our simulations is reminiscent of predictions that ecological speciation with gene flow proceeds rapidly once a threshold number of divergently selected mutations has accumulated (Flaxman et al. 2014; Feder et al. 2014). Flaxman et al.'s (2014) simulations of this process found that it occurred under a wide range of mean selection coefficients and migration rates. However, they also cautioned that scenarios other than speciation with gene flow could produce such a pattern (Flaxman et al. 2014; Feder et al. 2014). We found some evidence for a "congealing"-like process in simulations with divergent selection and high recent gene flow, in that d_{xy} exhibited a rapid increase between 4 and 8 million generations. Further examination will be needed to determine under what conditions this pattern occurs and whether it reliably indicates divergence with selection and gene flow. However, nonlinear growth of F_{ST} outlier regions in many scenarios that lacked selection indicates that this pattern cannot be attributed to "congealing"-like processes. While Flaxman et al.'s (2014) results and ours are not directly comparable—the former used an individual-based forward simulation of only loci that differed between species while we employed a coalescent simulation of a fixed genome size—both studies suggest that characterization of "congealing"-like processes in allopatrically diverging populations, both with and without selection, is the next step in the maturation of speciation genomics as a field. Theoretical work like this is important for understanding emerging results from empirical studies of genomic divergence across multiple speciation events.

Outlier loci are detected in many ways (e.g. Turner et al. 2005; Andrew and Rieseberg 2013; Gagnaire et al. 2013; Renaut et al. 2013; Soria-Carrasco et al. 2014). Our method—using an arbitrary high percentile of the F_{ST} distribution of all windows—is a particularly rough heuristic, in that it finds areas of the genome that should be enriched for loci under divergent selection, but it will do so even if no selection has occurred, as in these simulations. Since applying this method to data from wild populations will detect both selected and neutral loci, it is necessary to understand how the method treats purely neutral loci. Additionally, our simulations that included divergent selection only modelled selection on a single locus and, due to computational limits, only examined a small (100 kb)

neutral region linked to it. More complex genetic architectures of selected loci could produce radically different patterns of genomic divergence, but would require a different modelling approach to simulate.

The choice of F_{ST} outlier threshold is arbitrary, and we found qualitatively similar results with different thresholds (Figure A3). What is key for our purposes is that we set the same absolute threshold for all comparisons between species (Kronforst et al. 2013). Setting a different threshold for each species pair would remove the effects of divergence time on F_{ST}, and thus make it impossible to study the relationship between divergence time and the proportion of loci in highly diverged regions, the sort of study required to look for genome-wide congealing (Feder et al. 2014). Other recent studies have compared the position of outlier regions among parallel species pairs in different geographic contexts and gene flow scenarios, largely without considering divergence time (Roesti et al. 2012; Gagnaire et al. 2013; Renaut et al. 2013, 2014; Soria-Carrasco et al. 2014). In such comparisons, setting separate thresholds for each species/population pair is appropriate. However, to look for changes in the proportion of the genome that is highly divergent over time, it is necessary to set a single threshold.

Our findings of an exponential increase in low gene flow scenarios may follow from applying a single threshold. Statistically, applying a single threshold to the extreme tail of a pool of overlapping normal distributions results in an exponential increase, because this combined tail is dominated by values from the distributions with the furthest offset means (in our case, the older species pairs; Figure A9). This phenomenon may thus underlie our findings for low gene flow scenarios (Figures A5, A7). However, high gene flow alters the variance and/or skew of the F_{ST} distributions and reduces the offset among their means (Figures A6, A8), producing different patterns of outlier region growth.

In our simulations, each species pair was subject to the same demography, gene flow, and selection scenario, differing only by divergence time. Having different pairs of species experience different amounts and timing of gene flow, allowing changes in population sizes, and incorporating other more complex demographic histories and variation in recombination rates (including variation within a genome) would further change these distributions, and make the relationship between gene flow and the size of the divergent regions less clear. Different durations of gene flow are likely to have

a large impact on the growth of outlier regions because of gene flow's homogenizing effect in the absence of selection. While methods exist to determine the demographic history of related populations (Nielsen and Wakeley 2001; Hey and Nielsen 2004; Becquet and Przeworski 2009), and one could then simulate neutral evolution based on that demography to which real data could be compared, such simulations are computationally intractable for large linkage groups. Likewise, taxa with different tree topologies would require corrections for phylogenetic nonindependence before these comparisons could be made (Felsenstein 1985). Nonetheless, examining the raw distributions of F_{ST} per window could allow greater insight in more complicated demographic scenarios.

Our findings suggest some first steps for examining divergence at a genome-wide scale in comparative studies. The growth of outlier regions provides information about whether selection acted during speciation only under some circumstances, specifically when extensive gene flow is known to have occurred. This pattern combined with changes in d_{xy} between species pairs warrants further study as an indicator of speciation with gene flow. Finally, our findings reinforce the fact that not all patterns found in species that are known to experience divergence with gene flow and/or selection are characteristic of divergence with gene flow or selection. To make such claims, null models are necessary for comparison, but they require estimates of demography (which may be unreliable) and massive computational power. Refinement of these techniques offers great promise to look at genome-wide changes during speciation.

Acknowledgments

We thank J. Ross-Ibarra for initiating our interest in this topic; R. Márquez Pizano and M. Michalska-Smith for providing invaluable help with the simulations; and P. Nosil and four anonymous reviewers for helpful feedback that greatly improved the manuscript. The authors are supported by grants from the Natural Sciences and Engineering Research Council of Canada, the Smithsonian Institution, the National Science Foundation, and the National Institutes of Health.

CHAPTER 3

FEMALE MATE CHOICE IS A REPRODUCTIVE ISOLATING BARRIER IN *HELICONIUS* BUTTERFLIES

This chapter was first published as "Southcott, L. and M. R. Kronforst, 2018. Female mate choice is a reproductive isolating barrier in *Heliconius* butterflies. Ethology, 124:862-869" and is reproduced here with permission. Supplemental material referred to in this chapter is included as Appendix B, "Supplemental material for Chapter 3". Data and code for statistical analyses are archived in the Dryad data repository (Southcott and Kronforst 2018).

Introduction

SPECIATION has produced the astounding variety of organisms that so fascinate biologists. In sexually reproducing organisms, speciation is the evolution of barriers to gene flow, creating independent lineages out of previously connected populations (Coyne and Orr 2004). Of the many barriers that can prevent interbreeding, those that occur prior to mating can exert a relatively large influence on total reproductive isolation: though hybrids may be sterile, strong premating isolation prevents them from being formed at all (Schemske 2000; Ramsey et al. 2003). Premating barriers are especially important in cases of secondary contact or speciation with gene flow (Abbott et al. 2013).

In insects, many mechanisms can cause premating isolation: *Rhagoletis* flies mate on their host plants, so host plant preferences are a substantial barrier to hybridization (Powell et al. 2012). Damselfly species often differ in genital shape, creating a tactile cue that enables females to reject heterospecific males (McPeek et al. 2011). Songs of male *Laupala* crickets match the preferences of conspecific females (Wiley and Shaw 2010). Female, and sometimes male, preference underlies behavioural isolation between many pairs of *Drosophila* species (Coyne and Orr 1989; Noor 1995; Jennings et al. 2014), and these preferences can even be learned (Dukas and Scott 2015).

The genus *Heliconius*, containing about 45 species of Neotropical butterflies, has featured promi-

nently in speciation research over the past three decades. They are relatively easy to rear in captivity, show geographic variation in aposematic wing colour pattern within species, and mimic both congeners and more distantly related butterfly species. *Heliconius* butterflies mate assortatively based on several cues, especially wing colour pattern (Jiggins et al. 2001; Kronforst et al. 2006b; Merrill et al. 2014). Interspecific matings produce hybrid offspring that may be sterile or more vulnerable to predators because they do not match either aposematic parental species (Naisbit et al. 2002; Merrill et al. 2012). Unlike in many taxa, male choice has been much more commonly studied than female choice in *Heliconius*, because male choice is easier to test with model females as stimuli and because male choice is the first step in mating interactions. Female choice studies have only documented mate preference for variation (natural or experimentally-induced) in conspecific males (Finkbeiner et al. 2017; Chouteau et al. 2017; Darragh et al. 2017). However, males still regularly court heterospecific females when they have the opportunity (Merrill et al. 2011a) and researchers have generally found stronger assortative mating between species when there is the potential for female choice in the experimental design (Mérot et al. 2017). Furthermore, females are likely to bear a high cost if they hybridize, because they have low remating rates (Walters et al. 2012) and thus, lower-fitness hybrids would make up most of their offspring. Therefore, female choice could facilitate speciation within the genus. The traditional focus on male mate preference in *Heliconius* research may mean we are missing a piece of the puzzle in understanding the origin and maintenance of species in this genus. Here, we present an experiment to determine whether female *H. cydno* prefer males of their own species to males of the closely related *H. pachinus* (Figure 3.1).

Methods

Butterflies

Heliconius cydno galanthus occurs on the Caribbean coast of Central America from western Panama to southern Mexico. *Heliconius pachinus* is restricted to the Pacific coast of Costa Rica and Panama (Rosser et al. 2012). The two species diverged approximately 430,000 years ago (Kronforst et al. 2013).

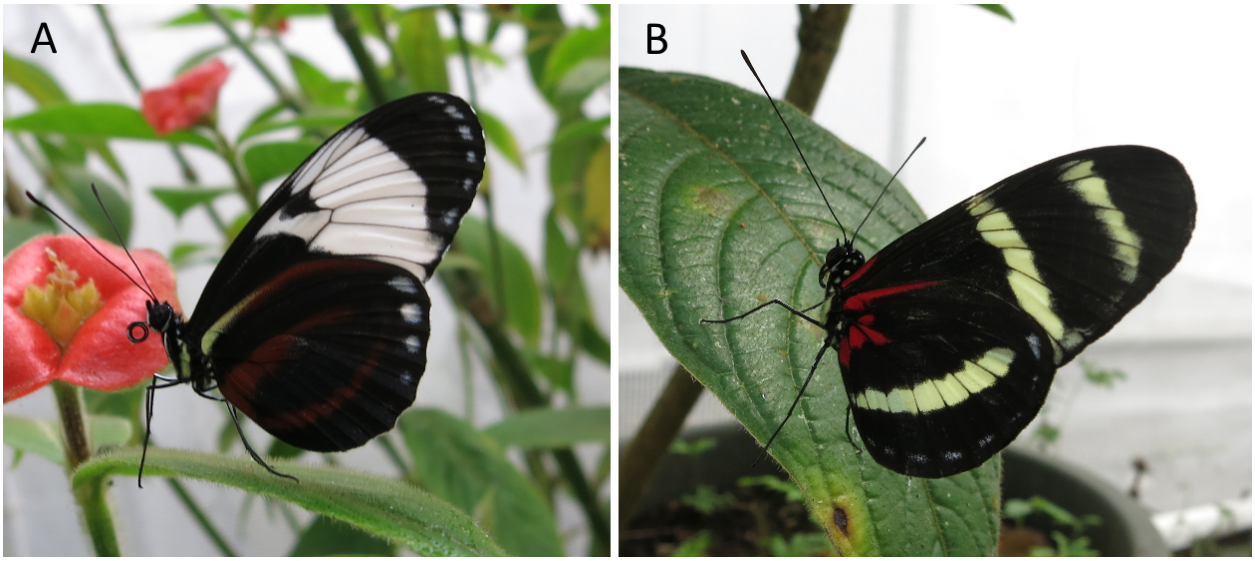

Figure 3.1: Butterfly species used in this study. A: *Heliconius cydno galanthus*. B: *Heliconius pachinus*.

There is ongoing gene flow primarily from *H. pachinus* into *H. cydno* (Kronforst et al. 2013, 2006a), and hybridization is probably most prevalent around San Jose, Costa Rica, where butterflies can cross the central mountain range through a lower elevation plateau (Kronforst et al. 2007). Male mate choice contributes to reproductive isolation between the species (Kronforst et al. 2007, 2006b), but is incomplete, with 12 of 75 recorded matings in Kronforst et al. (2007) being interspecific.

The butterflies used in our experiments came from captive populations we established and maintained at the Smithsonian Tropical Research Institute's insectaries in Gamboa, Panama. The captive *H. cydno* population was founded with approximately 15 wild individuals from Turrialba, Costa Rica in September 2015. The *H. pachinus* population came from eight butterflies from Reserva Forestal El Montuoso, Herrera, Panama, caught in February 2016, with 15 additional wild-caught butterflies added in April 2017. Experiments took place between May 2016 and August 2017.

Adult butterflies were kept in 2.8 x 2.7 x 1.8 m cages separated by species and sex and provided with a sugar-water solution and flowers of *Lantana camara*, *Psychotria poeppigiana*, *Gurania eriantha*, *Psiguria triphylla*, and/or *Psiguria warscewiczii* daily as a pollen source. Caterpillars of both species were raised on *Passiflora triloba* and *P. edulis* plants until pupation.

Mate choice experiment

To test whether naive virgin female *H. cydno* prefer to mate with conspecifics, we conducted a no-choice experiment in which a female was paired with either a *H. cydno* or a *H. pachinus* male and thus given the opportunity to mate or not.

We painted females' wings yellow to increase the probability of *H. pachinus* males approaching them. Kronforst et al. (2006b) found that *H. pachinus* males were as likely to approach wings of *H. cydno* females from a line that had a yellow forewing band introgressed from *H. melpomene* as they were to approach wings of *H. pachinus* females. We chose the simpler method of painting the forewing band to avoid the potential effects of inbreeding and *H. melpomene* genetics on female behaviour. On the day of their emergence and after their wings had fully dried, we used a Copic YG21 Anise paint pen on the dorsal surface of the forewing. This paint dries rapidly and females can fly normally within seconds of its application. Females to be paired with *H. pachinus* males had their white forewing band painted yellow, while females to be paired with *H. cydno* males had paint applied to the black part of the forewing (approximately equal area to the white band) as a control (Figure B1). Spectrophotometry indicated that painting over the black part of the wing did not substantially change its reflectance spectrum, while the yellow paint on the white band was a close approximation of the yellow pigment of *H. pachinus* and other *Heliconius* species (Figure B2). A pilot study found no difference in survival or activity levels between painted and unpainted females (Figure B5).

Typical courtship in adult-mating *Heliconius* begins with a male approaching a perched or flying female. The male chases a flying female, sometimes appearing to touch her, until the the male breaks off pursuit or the female lands. Courtship continues with the male hovering over the perched female, possibly to waft pheromones produced by specialized androcondial scales on the wings, towards the female (Darragh et al. 2017). The male will attempt to land next to the female, facing in the same direction, and bend his abdomen towards hers to attempt to begin mating. Less commonly, males may land facing the female and touch her head with their proboscis; the function of this behaviour is unknown. Perched females may execute several behaviours during courtship. They may walk or fly away from the male; hold the wings open, preventing the male's abdomen from reaching hers; or keep

the wings closed, allowing mating to occur. She may also flutter her wings and evert her abdominal scent glands; this may be a rejection behaviour, especially in females who have previously mated. More detailed accounts of *Heliconius* courtship are given in Crane (1955, 1957); Klein and De Araújo (2010); Jiggins (2016).

Table 3.1: Behaviours recorded during no-choice experiment

Behaviour	Description	Type
Males		
Chase	Male follows female closely while both are flying	Courtship
Hover	Male hovers over perched female	Courtship
Mate attempt	Male lands next to female and bends abdomen towards hers	Courtship
Females		
Open wings	Perched female opens wings and holds them there	Rejection
Flutter	Perched female rapidly opens and closes wings while lifting abdomen and, usually, exposing abdominal scent glands	Rejection
Close wings	Perched female holds wings closed	Acceptance
Fly	Female flies away from male (including taking off from a perched position)	Rejection

Experimental females were housed overnight in a large cage with other virgin females. Females were tested either one or two days after emergence, when they are most receptive to mating and when mating typically takes place in the wild (Jiggins 2016). A stimulus male - either *H. cydno* or *H. pachinus* at least 10 days post-emergence - was isolated in the experimental cage the day before the experiment. On the day of the experiment, the female was placed in a popup cage (30 x 30 x 30 cm) in the experimental cage for 5 minutes to acclimate. The female was then released, and both the male's courtship attempts and the female's responses were recorded until mating occurred or for up to 2 hr. We selected 3 male and 4 female behaviours to record based on how commonly they occur during courtship, how easily an observer can score them, and how likely they are to be correlated with the courtship's outcome (mating or not mating). Table 3.1 describes the male and female behaviours recorded. Behaviours were recorded every minute, so repeated instances of the same behaviour during the same minute were not counted, but instances of two or more different behaviours during the same minute were counted. Each female and each male was used in only one experiment to ensure the independence of trials.

Statistical analysis

We tested whether interspecific or intraspecific pairs mated more often with a chi-squared test with Yates' continuity correction. The outcome (mating or not mating) of a no-choice trial could be attributed to male choice, female choice, or both. We tested whether males of the two species courted females equally often with Mann-Whitney U tests on the total number of courtship behaviours in a trial, the number of chases or hovers, and the number of mating attempts, excluding trials in which the male never courted. To confirm that male courtship rate did not predict the outcome of the trial, we conducted a logistic regression (GLM with a logit link function) with male species and number of courtships (the sum of all male behaviours) as independent variables and the trial outcome as the dependent variable. We examined whether females' behaviours per male courtship predicted the outcome of the experiments using logistic regression on only the data from intraspecific trials (there were not enough interspecific matings to test whether male species interacted with these behaviours). Finally, we tested whether female behaviour rates differed between inter- and intraspecific trials using Mann-Whitney U tests to determine whether females responded differently to different species of males. All analyses were performed in R (R Core Team 2013).

Results

Intraspecific pairs mated significantly more often than interspecific pairs (Table 3.2, χ^2 excluding trials with no courtships: $\chi^2 = 9.28$, df = 1, p = 0.002). *Heliconius pachinus* males were more likely to ignore the female altogether: we excluded 21 trials with *H. pachinus* males because they performed no courtship behaviours, compared to 5 such trials for *H. cydno*. We excluded trials in which the male never courted from all subsequent analyses because there is no opportunity for females to exercise choice in this context.

The total numbers of courtship behaviours performed by male *H. cydno* and *H. pachinus* did not differ significantly (U = 338, p = 0.184; Figure B3). Numbers of hovers or chases were combined for analysis because the two behaviours were not recorded separately in some trials; the combined be-

Table 3.2: Outcomes of no-choice experiment

	Mating	No Mating	No courtship*
Interspecific	4	21	21
Intraspecific	15	9	5

*Trials in which males never courted the female were excluded from further analyses.

haviours did not differ significantly between male species (U = 292.5, p = 0.73, Figure B3). However, *H. pachinus* males performed significantly fewer mating attempts (U = 460, p = 0.000029, Figure B3). In a logistic regression of outcome (mating or not mating) vs. male species, number of courtships, and their interaction, the number of courtship attempts did not predict the outcome of the experiment (likelihood ratio tests of coefficients in a logistic regression: number of courtships p = 0.54; interaction between male species and number of courtships p = 0.57). Furthermore, comparing the full model to a reduced model with only male species as predictor, the reduced model had lower AIC (ΔAIC = 3.31) and a likelihood ratio test found that adding number of courtships did not improve the model (p = 0.71). Thus, we attribute the difference in mating rates to female preference for conspecific males rather than different intensity of male courtship once non-courting males were excluded.

In intraspecific trials, "close wings" behaviour was positively correlated with the outcome of the experiment, suggesting that wing closing indicates female acceptance of a courting male (coefficient = 14.3, SE of coefficient = 5.1, p = 0.0088). The other three behaviours were not significantly correlated with outcome, though all had negative coefficients and are considered rejection behaviours by other authors (Figure 3.2, Table 3.3; Jiggins 2016; Chouteau et al. 2017). The rates of wing opening and fluttering did not differ significantly between interspecific and intraspecific trials, although both were performed more towards *H. pachinus* males. Females closed their wings more often in intraspecific trials and flew away from the male more often in interspecific trials (Table 3.4, Figure 3.3).

Discussion

Intraspecific no-choice trials ended in mating much more often than interspecific trials did. The lack of difference in courtship rates between species among males who courted at least once strongly

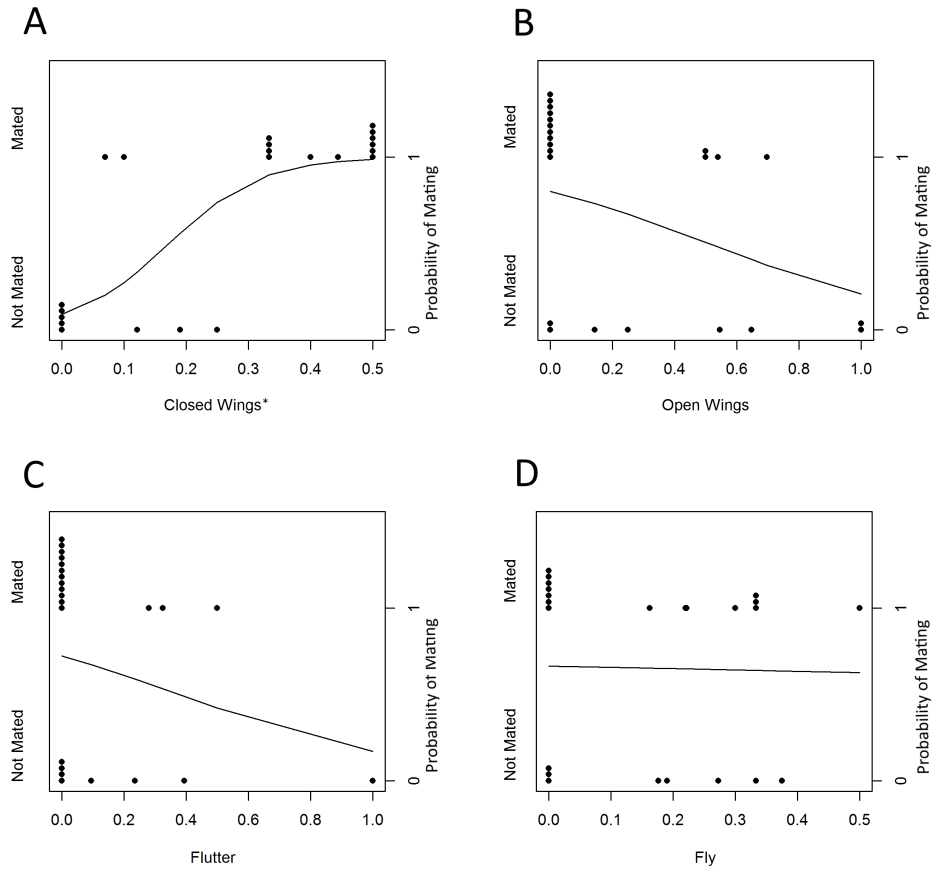

Figure 3.2: Outcome of no-choice trial and number of female behaviours per male courtship in intraspecific trials. Lines and right *y*-axis: Probability of mating versus female behaviour from GLMs. A: closed wings. B: open wings. C: flutter. D: fly. See Table 3.1 for descriptions of behaviours. Asterisks indicate behaviour that was significantly correlated with the trial's outcome in a logistic regression.

suggests that female choice determined the outcome. The differences in female response behaviours (closing wings and flying away) to different species of courting males further suggests that females actively chose mates. This is the first demonstration of interspecific female choice in *Heliconius* butterflies, a model genus for speciation research with extensive evidence of male mate choice. Although male choice exists between these species (Kronforst et al. 2006b), it is weak enough that, with assistance from the manipulated female wing colour, we could observe sufficient interspecific courtships to examine females' response to heterospecific males.

Our study adds to other attempts to document female mate preference in *Heliconius*. Recent studies have revealed intraspecific female choice in several *Heliconius* species using a variety of meth-

Table 3.3: Results of logistic regressions of female behaviours per male courtship in intraspecific trials vs. trial outcome (mating or no mating)

Behaviour	Coefficient	SE	p
Closed wings	14.3	5.1	0.0088
Open wings	-2.74	1.5	0.062
Flutter	-2.55	2.1	0.22
Fly	-0.32	2.8	0.91

Table 3.4: Results of Mann-Whitney U tests comparing female behaviours per male courtship between trials with *H. cydno* (intraspecific) and *H. pachinus* (interspecific) males.

Behaviour	U	p
Closed wings	369.5	0.001
Open wings	239	0.56
Flutter	200.5	0.12
Fly	140.5	0.0087

ods. All suggest that females exert choice during courtship based on multimodal signals, particularly vision and olfaction. In *H. erato*, females approach moving paper wings more often when they are UV reflective and have the appropriate yellow pigment (Finkbeiner et al. 2017). In the polymorphic species *H. numata*, females perform more rejection behaviours towards moving models made of dead males' wings if those wings are of the same colour pattern morph as the female (Chouteau et al. 2017). Backcross hybrid females between *H. cydno* and *H. melpomene* are less likely to mate when they are heterozygous at the locus controlling a colour pattern element on the hindwing than when homozygous (Merrill et al. 2011b). In *H. timareta*, *H. erato*, and two subspecies of *H. melpomene*, females are less likely to mate with males whose pheromone-producing androconial scales have been blocked with nail varnish than with non-blocked males (Darragh et al. 2017). Similarly, perfuming conspecific males with another species' pheromones reduced the likelihood of females mating with them (Mérot et al. 2015). Because both visual and olfactory cues differ among *Heliconius* species, these same cues could be used in interspecific female mate choice. *Heliconius cydno* and *H. pachinus*, however, have minimal differences in male pheromone composition (Schulz et al. 2007; Estrada et al. 2011), so females may be more likely to choose between these species based on visual and other non-olfactory cues.

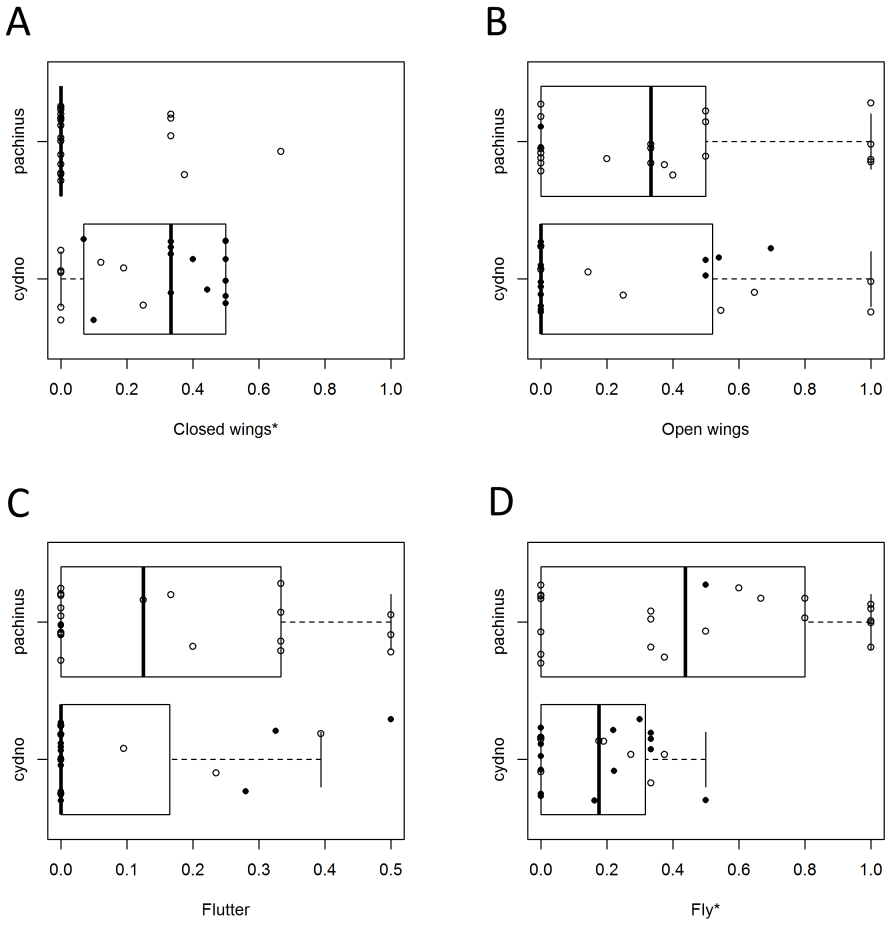

Figure 3.3: *Heliconius cydno* female behaviour rates in trials with *H. cydno* and *H. pachinus* males. A: closed wings. B: open wings. C: flutter. D: fly. Asterisks indicate behaviours whose frequency differed significantly between intraspecific and interspecific trials. Black dots: trials that ended in mating. White dots: trials that did not end in mating. Some sample sizes differ from those in Table 3.2 because not all behaviours were recorded in a few early trials.

Female choice acts within and between other butterfly species. In *Pieris occidentalis*, females prefer males of their own species over male *P. protodice*, and increasing the area of melanized spots on the forewing of *P. protodice* males increases the rate at which *P. occidentalis* mate with them (Wiernasz and Kingsolver 1992). A series of experiments revealed that *Colias philodice* females prefer conspecific males over *C. eurytheme* males, but that wing colour alone does not affect their preference (Silberglied and Taylor 1978). Females of the cryptic species *Leptidea sinapis* and *L. reali* use long courtships to distinguish between males, which court both species indiscriminately (Friberg et al. 2008). Other studies have not tested interspecific mate choice directly, but have manipulated conspecific male phe-

notypes. They include studies of eyespot morphology and pheromones in *Bicyclus anynana* (Robertson and Monteiro 2005; Costanzo and Monteiro 2007) and of ultraviolet reflectance, iridescence, and other visual cues in *Pieris rapae* (Morehouse and Rutowski 2010), *Battus philenor* (Rutowski and Rajyaguru 2013), and *Hypolimnas bolina* (Kemp 2007), among other species.

While female choice acts in both inter- and intraspecific contexts in many butterflies, it is not always clear how much such choice contributes to total reproductive isolation. In many species, mate choice is mutual, but it is also often sequential, with males choosing whether to approach a female before the female can choose to accept or reject a male. This is certainly the case in *Heliconius*, and has long complicated efforts to detect female choice (Merrill et al. 2015). *Heliconius cydno* and *H. pachinus* are one of the younger species pairs within the genus (approximately 430 kya divergence; Kronforst et al. 2013). Compared to *H. cydno* and its next closest relative *H. melpomene*, which diverged approximately 1.4 mya (Kronforst et al. 2013), male *H. cydno* and *H. pachinus* are more likely to engage in heterospecific courtships (Merrill et al. 2011a; Mérot et al. 2017). This weaker male choice made it possible for us to induce *H. pachinus* males to court *H. cydno* females in sufficient quantities to test female choice. However, it also suggests that later in speciation between non-co-mimics female choice may decrease in relative importance among isolating barriers because male choice is strong enough that females are seldom courted by heterospecific males. Nevertheless, in young species pairs such as the one we studied, mate choice by both sexes contributes to reproductive isolation.

We have demonstrated for the first time that interspecific female mate choice is a reproductive isolating barrier between two *Heliconius* species. This finding parallels recent research showing intraspecific female choice in several *Heliconius* species and adds to the indirect case for interspecific female choice, filling a longstanding gap in the extensive literature on speciation and hybridization in this genus. Further research on the cues females use to select mates and whether they are linked to divergently selected traits is needed to understand the role of female choice in speciation.

Acknowledgments

We thank Owen McMillan and the staff of the Smithsonian Tropical Research Institute for allowing us to use the insectary facility. We also thank Oscar Paneso, Emma Tomaszewski, Aaron Goodman, Holly Black, Amber Antonison, Lina Melo, Diego Vergara, Chantelle Doyle, and Liz Evans for capable and enthusiastic assistance with rearing butterflies, maintaining the insectaries, and performing experiments, and the rest of the STRI Gamboa community for their logistical and emotional support. We are also grateful to Trevor Price, Jill Mateo, and Corrie Moreau for helpful comments on this manuscript. This work was supported by the Society for the Study of Evolution (Rosemary Grant Award to LS); the University of Chicago (Center for Latin American Studies Tinker Field Research Grant and Hinds Award to LS); the Smithsonian Institution (Short-term Fellowship and CIC Graduate Fellowship to LS); National Science Foundation (grant number IOS-1452648 to MRK); and National Institutes of Health (grant number GM108626 to MRK).

CHAPTER 4

PREMATING REPRODUCTIVE ISOLATION IN *HELICONIUS* BUTTERFLIES

Supplemental material referred to in this chapter is included as Appendix C, "Supplemental material for Chapter 4".

Introduction

PREMATING reproductive isolation, or a decrease in gene flow due to preference for conspecific mates, plays a large role in speciation (Schemske 2000). It can allow persistence of incipient species by preventing hybrid formation and complete speciation through reinforcement.

Premating isolation can evolve by reinforcement—when hybrids are less fit than parental genotypes and there is thus selection for assortative mating to avoid hybrid formation. However, this idea was controversial until the detection of stronger premating isolation between sympatric than allopatric populations of *Drosophila*, which suggested that reinforcement is common in nature (Coyne and Orr 1989, 1997). Part of the theoretical objections to reinforcement—and to selection for assortative mating in general—was that mate preference is easily separated from postmating isolation traits and traits involved in signalling to mates by recombination when hybrids are formed. Therefore, premating isolation evolves most readily when there is a strong genetic correlation between the loci underlying assortative mating and the loci underlying mate choice cues (Felsenstein 1981; Noor 1999; Ortiz-Barrientos et al. 2009; Servedio 2009). This prediction is difficult to test but a few examples fit the pattern (e.g. Noor 1995; Nosil et al. 2003; Wiley et al. 2011; McNiven and Moehring 2013; Pfennig and Rice 2014), including genetic correlations between mate preference and wing colour patterns (which are mate choice cues) in *Heliconius* butterflies (Kronforst et al. 2006b; Merrill et al. 2011b, 2019).

Heliconius butterflies have been studied for decades and have considerable light to shed on the evolution of interspecific mate preference. Many species/subspecies pairs have been tested to study aspects of mate preference like choice based on wing colour patterns (Jiggins et al. 2001; Kronforst et al. 2006b; Merrill et al. 2014; Finkbeiner et al. 2014, 2017), choice based on pheromones (Mérot et al. 2015; Darragh et al. 2017), accidental courtship between co-mimics (Estrada and Jiggins 2008), and reinforcement (Kronforst et al. 2007).

In this study, I measure interspecific male mate preference in *Heliconius cydno galanthus*, *H. pachinus*, and their F1 and F2 hybrids. I compare these results to those of a previous study and consider the statistical power of my study by simulating experiments with different underlying preferences. I then put these investigations in the context of the genus *Heliconius* by conducting a meta-analysis of interspecific/inter-subspecific mate choice studies.

Methods

Butterflies

I established captive populations of each species at the Smithsonian Tropical Research Institute's insectaries in Gamboa, Panama. Wild *H. cydno* were collected in Turrialba, Costa Rica and approximately 15 individuals were used to found the captive population. *Heliconius pachinus* were collected around Reserva Forestal El Montuoso in Panama and 8 individuals established the captive population. The *H. pachinus* population was supplemented with wild-caught individuals from the same site on two occasions; the *H. cydno* population had no additional input.

Butterflies were kept outdoors in 1.8 x 2.7 x 2.8 m cages separated by sex. Adults were provided with a sugar-water solution as well as flowers of *Lantana camara*, *Gurania eriantha*, *Psiguria triphylla*, and/or *Psiguria warscewiczii* as pollen sources. *Psychotria poeppigiana* plants were grown in pots in the cages both as an additional pollen source and for shade and shelter.

Females were provided with *Passiflora menispermifolia* plants for egg-laying. Eggs were collected daily and kept in small cups which were checked daily for hatching. Hatchling caterpillars were trans-

ferred to whole *Passiflora platyloba* or *P. edulis* plants or cuttings. Late fifth instar caterpillars were transferred to smaller parasitoid-proof popup cages for pupation.

Five captive-reared butterflies of each species have been deposited as voucher specimens in the insect collection of the Field Museum of Natural History. The *H. cydno* voucher specimens are catalog numbers FMNH-INS 2994222-2994226 and the *H. pachinus* specimens are FMNH-INS 2994227-299423I.

Crosses

I established F1 crosses between the two species in both directions by hand pairing or leaving a male and female in the same cage until they mated. F1 individuals from the same family were likewise paired to form F2 crosses. Mated females were kept in 1.5 x 1.5 x 2 m cages with a *Passiflora menispermifolia* plant for egg laying. Eggs, larvae, and pupae were handled as described above.

Mate choice experiments

I conducted dichotomous choice trials, in which a single male chooses between two females presented simultaneously, to assess the preference for *H. cydno* or *H. pachinus* females of *H. cydno*, *H. pachinus*, F1 hybrid, and F2 hybrid males. All males were at least 10 days old on the day of the experiment to ensure that they were sexually mature and producing pheromones and had been kept in a cage without access to females.

Experiments were conducted in a 1.5 x 1.5 x 2 m cage with *Lantana camara* flowers, sugar water, and a *Psychotria poeppigiana* plant. The day before the experiment, the male to be tested was placed in the cage to acclimate him to the testing environment. On the day of the experiment, one virgin female of each species, roughly matched for age, was selected. The females were held in a 30 x 30 x 30 cm popup cage within the experimental cage for 5 min before the trial started. They were then released simultaneously into the cage with the male. The male's interactions with each female were recorded until mating began or for up to 2 hours. Experiments were all conducted between 8:30 and 11:00 am. I recorded 4 male behaviours that indicate interest in females: approach (change flight

path to move towards the female), chase (pursuing the female in flight), hover (flying in place above a perched female), and mate attempt (landing next to the female and bending the abdomen towards hers). I also recorded whether mating occurred and with which species, and which species was courted first. Behaviours were recorded every minute, so repeated instances of the same behaviour during the same minute were not counted. If mating occurred, the butterflies were gently separated and the trial ended. Each male was tested only once. Females were reused (even if they had mated and been separated) as long as their wings were not badly worn.

Correlation between wing colour pattern and mate preference

I recorded the colour of each male's forewing (white or yellow) and hindwing (yellow/present, black/absent, or heterozygote) bands. Forewing band colour is controlled by the gene *aristaless1* on chromosome 1 (Westerman et al. 2018). The white (*H. cydno*) allele is dominant to the yellow (*H. pachinus*) allele and the colour of the band or bands is unlinked to the shape, size, or number of bands. Hindwing band presence is determined by the gene *cortex* on chromosome 15 (Nadeau et al. 2016) and the black/absent band (*H. cydno*) allele is partially dominant to the yellow/present (*H. pachinus*) allele; heterozygotes have a paler patch on an otherwise black hindwing.

Statistical analysis

I calculated a preference index for each male by dividing the number of behaviours towards the *H. cydno* female by the total number of male behaviours. To confirm that pure species males preferred conspecific females, I compared the preference indices of the two species with a Mann-Whitney U-test.

U-tests were also used to compare the preferences of (1) FIs of different cross directions, (2) F2s with white or yellow forewing bands, and (3) males who courted *H. cydno* vs. *H. pachinus* first. Kruskal-Wallis rank sum tests were used to compare the preferences of (1) different F1 families and (2) F2s with black, yellow, or heterozygote hindwing bands. All statistical analyses were performed in R (R Core Team 2013).

Modelling statistical power

I simulated preference data to determine whether the difference in preference of F2 males with white versus yellow forewing bars seen between the this study and that of Kronforst et al. (2006b) could be due to sampling error. I fit logit-normal distributions to the white forewing and yellow forewing preference data from Kronforst et al. (2006b) (Scenario 1) and from this study (Scenario 2) using the R package logitnorm (Wutzler 2018). I used this same package to simulate data drawn from those distributions for sample sizes of 20, 40, 60, and 80 butterflies. For each sample size, 75% of the samples had white forewings and 25% had yellow forewings in accordance with the Mendelian segregation pattern expected for the forewing band colour locus. I also simulated data for two other sets of distributions: one with a minimal difference between the preferences of individuals with white and yellow forewings (Scenario 3) and one with a moderate difference between them with yellow forewing males preferring *H. pachinus* females (Scenario 4). Each sample size was simulated ten times. The distribution parameters (μ and σ, the mean and standard deviation of the modelled variable's logit) for each scenario are presented in Table 4.1 and Figure 4.1 shows the density functions of these distributions.

Table 4.1: Parameters of the logit-normal distributions used to simulate preference data.

Scenario	Forewing colour	μ	σ
1. Kronforst et al. (2006b)	white	0.6	1.3
	yellow	-1.0	1.0
2. This study	white	0	1.0
	yellow	0.6	1.3
3. Minimal difference	white	0.2	1.1
	yellow	0.07	1.2
4. Small difference	white	0.4	1.2
	yellow	-0.46	1.1

Meta-analysis of *Heliconius* hybridization rates

I surveyed the literature on *Heliconius* mate choice to compare rates of interspecific or inter-subspecific mating throughout the genus. I included studies that reported results of experiments that used live

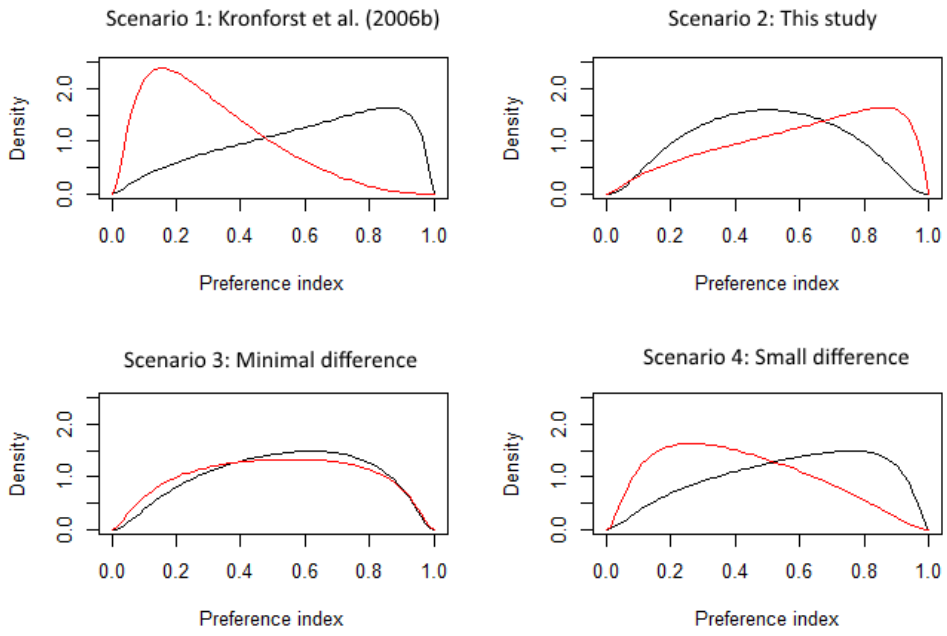

Figure 4.1: Logit-normal distributions representing four simulated preference scenarios. Red lines: *H. pachinus* males. Black lines: *H. cydno* males. Top left: Scenario 1, preference distributions similar to those observed by Kronforst et al. (2006b). Top right: Scenario 2, preference distributions similar to those observed in this study. Bottom left: Scenario 3, minimally different preference distributions. Bottom right: Slightly different preference distributions.

butterflies. Studies using model butterflies made of real wings or paper were excluded. I recorded the species or subspecies used; whether the pair was allopatric, parapatric, or sympatric; what metric was used to determine preference (mating or other courtship behaviour(s)); the sample size; the duration of the trials; whether the experimental design was no-choice, dichotomous choice of females, sequential presentation of females, or tetrad (one male and one female of each of two species); and sex ratio within trials. Published F_{ST} values between each species pair were also obtained from RAD-seq or whole genome sequencing data whenever possible. I calculated the hybridization rate for each species pair as either the proportion of trials resulting in heterospecific mating or the proportion of courtship events directed towards heterospecifics depending on the data available. Whenever possible, raw data (counts of courtships or mating events) were used, but in some cases only modelled likelihoods of courtships were available and in several others, I calculated hybridization as (1 - RI)/2 from values of reproductive isolation reported in Mérot et al. (2017). I conducted model selection with AICc using the exhaustive search function dredge in the R package MuMIn (Barton 2018) to

identify which fixed effects and two-way interactions best explained hybridization rate. Species pair was included as a random effect in all of these models. Table C1 in Appendix C lists the studies and species/subspecies pairs included in the meta-analysis.

Results

Mate preference

Pure species males preferred conspecific females ($U = 601.5$, $p = 0.00000015$, Figure 4.2). F1 males' preferences were skewed towards *H. pachinus* females, while F2 males' preferences were intermediate (Figure 4.2).

The F1 preference distribution did not differ significantly between cross directions (*H. cydno* mother or *H. pachinus* mother) ($U = 1006$, $p = 0.73$; Figure 4.3). It was also not significantly different among families (Kruskal-Wallis $\chi^2 = 0.2608$, $df = 2$, $p = 0.088$; Figure 4.4).

F2 male preference was not associated with the colour of the band(s) on the males' forewings ($U = 569.5$, $p = 0.26$; Figure 4.5) or hindwings (Kruskal-Wallis $\chi^2 = 0.352$, $df = 2$, $p = 0.84$; Figure 4.6).

The species identity of the first female courted in the trial was strongly correlated with the male's preference throughout the trial (Figure 4.7). Further, although preferences of 0 or 1 were rare for trials with more than 9 courtships across both parental species and hybrids (Figure 4.8), analyses of parental and F2 preference in a dataset with only trials with more than 9 courtships did not produce different results.

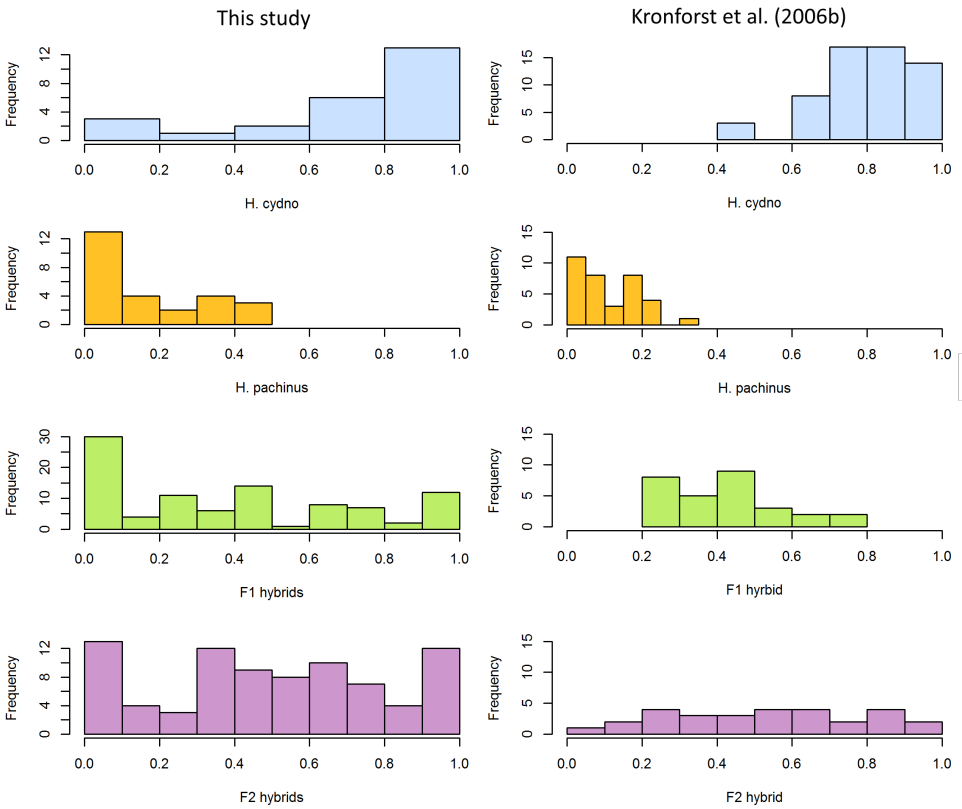

Figure 4.2: Mate preference index of pure species and hybrid males. A preference index of 1 indicates strong preference for *H. cydno* females.

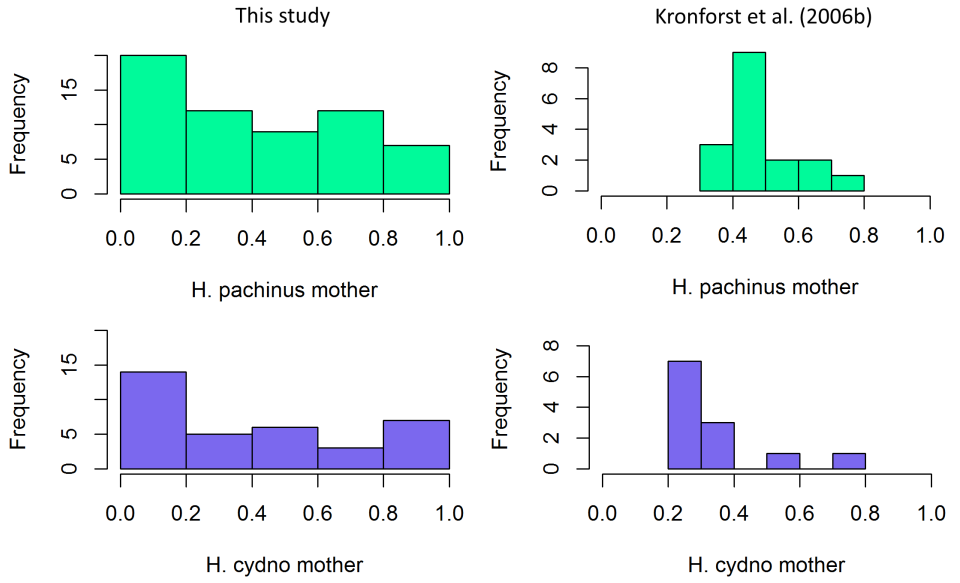

Figure 4.3: Preference index of F1 males by cross direction. Top: Males with *H. pachinus* mothers. Bottom: Males with *H. cydno* mothers.

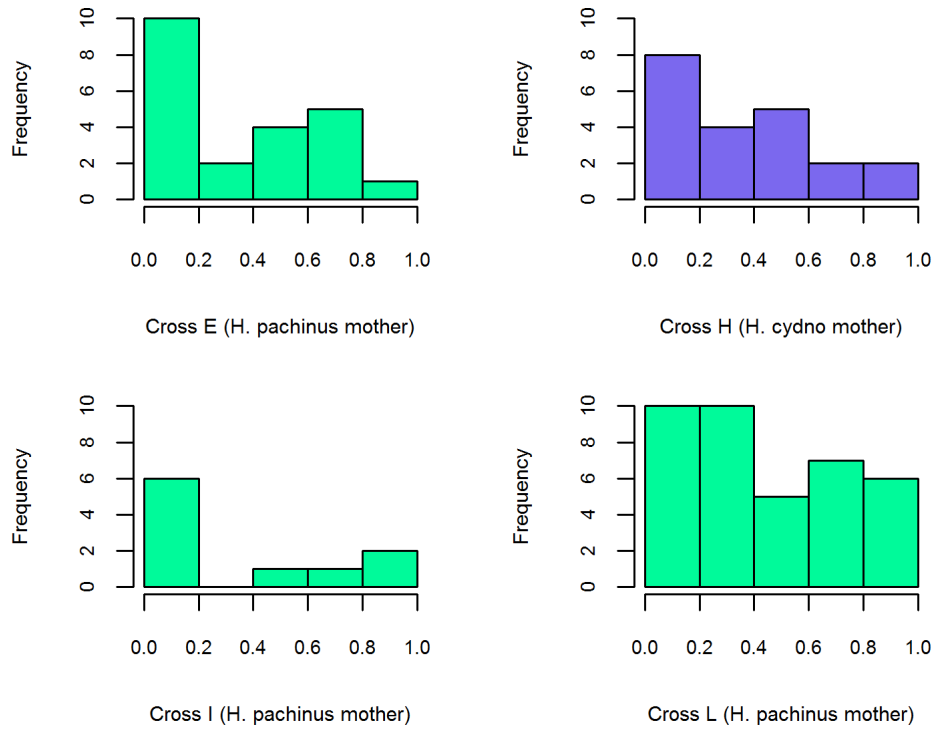

Figure 4.4: Preference index of F1 males by family. Crosses E, I, and L have *H. pachinus* mothers; cross H has an *H. cydno* mother. One other cross (*H. cydno* mother) had only 4 phenotyped offspring and is not shown.

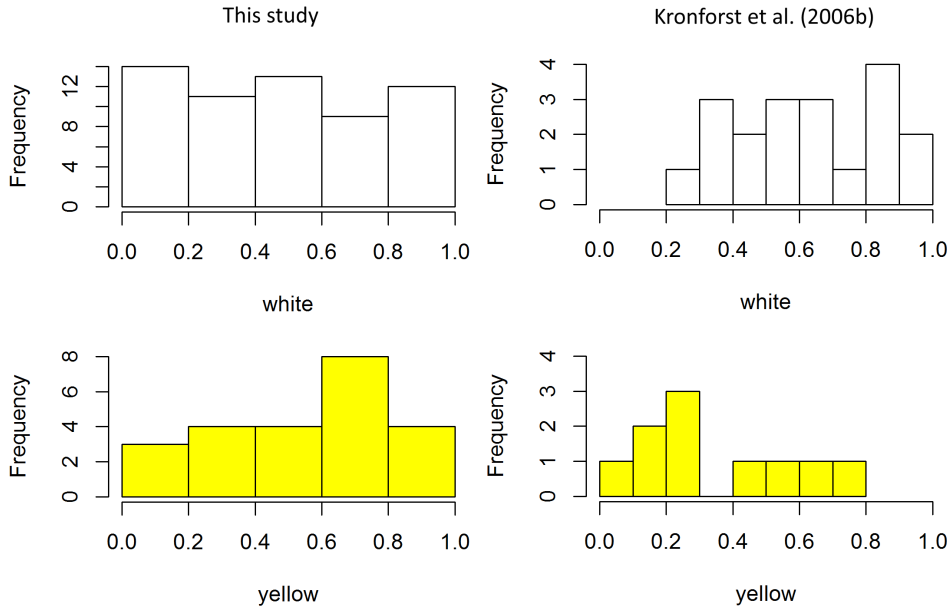

Figure 4.5: Preference index of F2 hybrid males by forewing band colour. Top: males with white forewing band(s). Bottom: males with yellow forewing band(s).

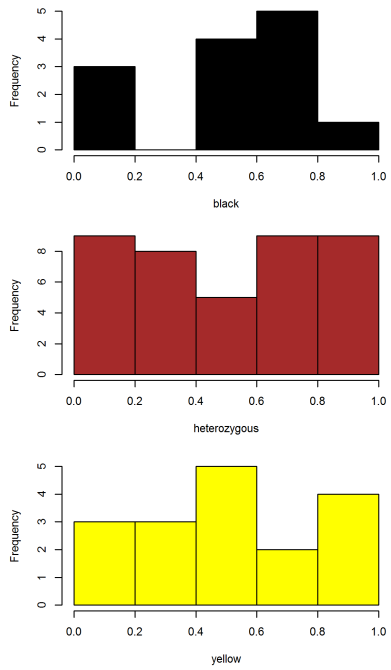

Figure 4.6: Preference index of F2 hybrid males by hindwing band colour. Top: males with black hindwing band. Middle: males with heterozygote hindwing band. Bottom: males with yellow hindwing band.

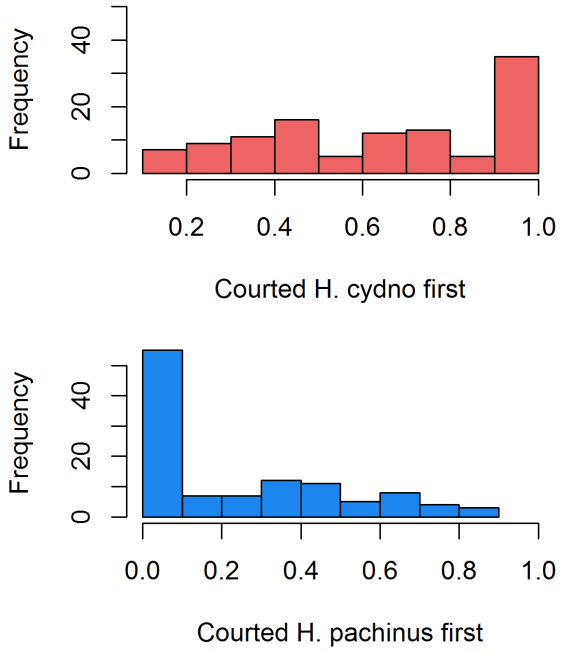

Figure 4.7: Preference index of all butterflies by the species of female first courted. Top: males that courted the *H. cydno* female first. Bottom: males that courted the *H. pachinus* female first.

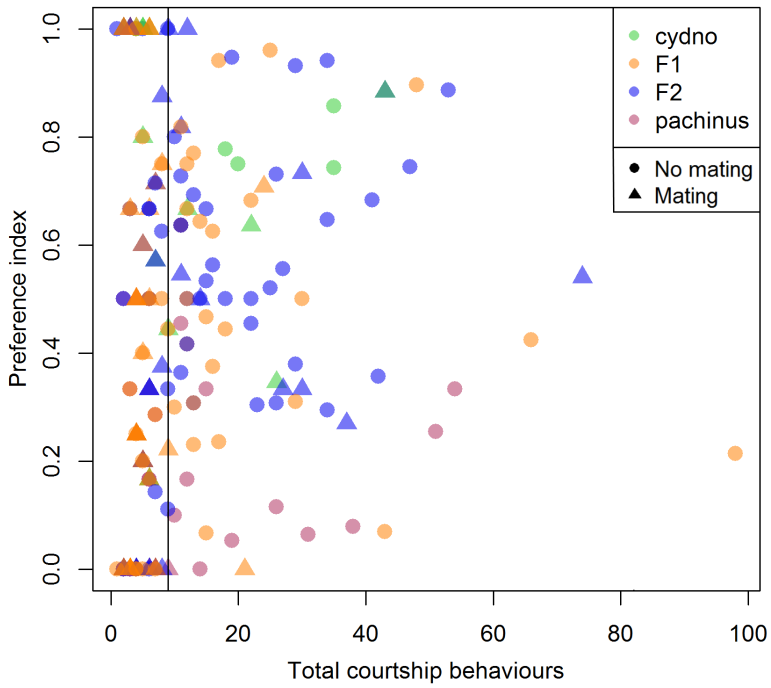

Figure 4.8: Preference index versus total number of courtships for all butterflies. Triangles indicate trials that ended in mating; circles, trials that did not end in mating. Vertical line indicates 9 courtship behaviours, the cutoff for reanalysis of the data.

Modelling statistical power

I simulated four preference scenarios for F2 males with different forewing colours, based on the two studies, no difference in preference, and a small difference in preference. If F2 males have a difference in preference comparable to that found by Kronforst et al. (2006b), then sample sizes of 40 and above nearly always found a statisically significant difference between white and yellow forewing males, and sample sizes of 20 found a statistically significant difference 6 out of 10 times (Figure 4.9). In a scenario where F2 males differed in preference on a scale comparable to this study (white forewing males have no preference, yellow forewing males slightly prefer *H. cydno*; Figure 4.1), a statistically significant difference was obtained at most in 6 of 10 simulations (Figure 4.9).

In Scenario 3, in which preference distributions were only slightly different between F2 male types, a statistically significant difference was found in at most 2 of 10 simulations. For the small difference in preference simulated in Scenario 4, the largest sample size simulations found a statistically significant difference in 9 of 10 runs, and for every sample size a significant result was obtained at least 4 of 10 times (Figure 4.9). For reference, this study had a sample size of 59 white forewing and 23 yellow forewing males, comparable to the largest sample size simulated. Kronforst et al. (2006b) tested 19 white forewing and 10 yellow forewing males.

Meta-analysis of *Heliconius* hybridization rates

17 studies testing 24 species/subspecies pairs were included in the meta-analysis. All but one tested both directions of each species pair, for a total of 67 measurements of premating reproductive isolation in *Heliconius*. Of these, 58 were within the cydno-melpomene-timareta clade, 6 were within the erato clade, and 3 were between the two clades. Roughly equal numbers of tests involved allopatric (20), slightly parapatric (18), largely parapatric (14), and sympatric (15) species pairs. A Kruskal-Wallis test found no significant differences in hybrid mating rates among distribution types (Kruskal-Wallis $\chi^2 = 6.57$, $p = 0.87$), but pairs with sympatric and largely parapatric ranges tended to have lower hybrid mating rates (Figure 4.10).

Most (44) experiments used mating as the indicator of preference; Mérot et al. (2017) compared

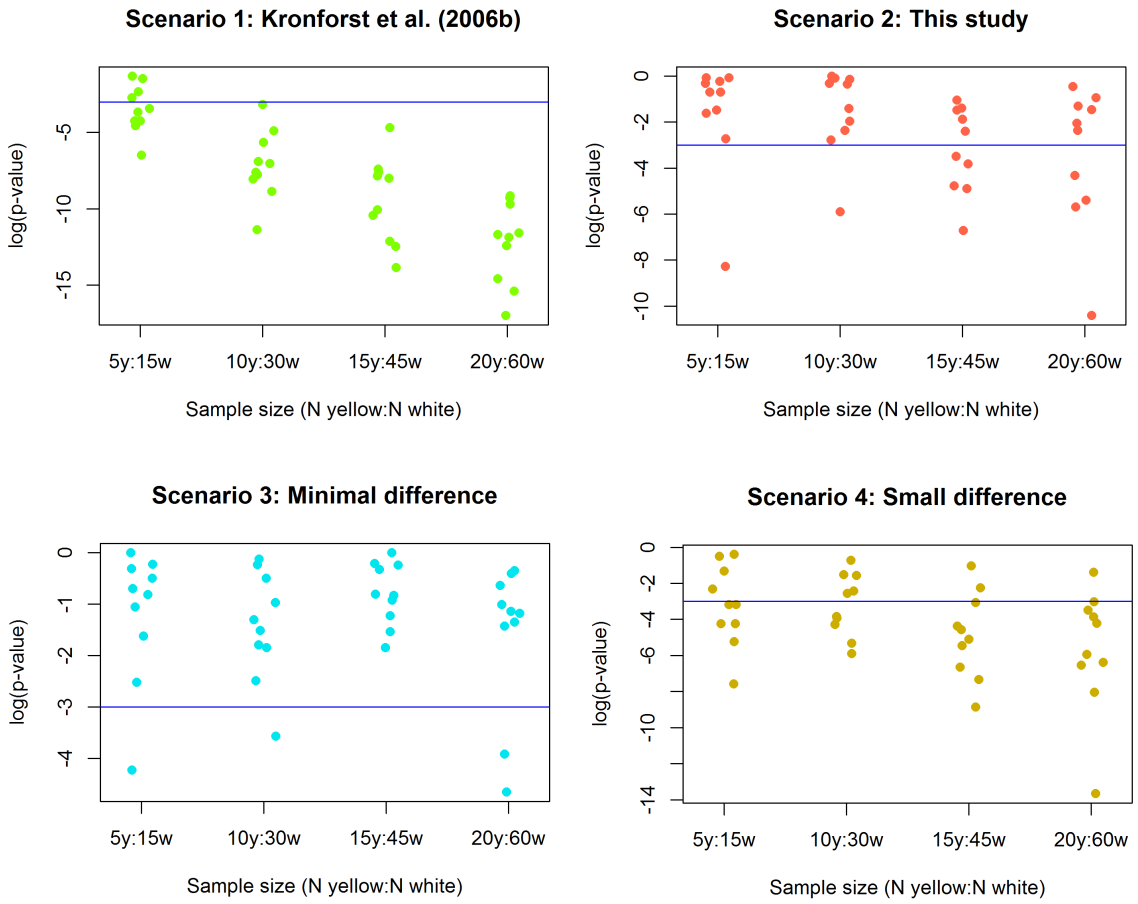

Figure 4.9: Log(p-values) from Mann-Whitney U-tests on simulated preference data. Horizontal blue lines indicate the threshold for significance at p = 0.05, so all points below the line represent statistically significant tests. For each scenario, four sample sizes were simulated ten times each (N = 20, 40, 60, and 80, with a ratio of 3 white: 1 yellow butterflies).

studies with model wings, live females (mating not allowed), and live females (mating allowed) and found that in most cases using mating and quantifying courtship behaviour both yielded similar estimates of reproductive isolation. Experiment duration varied from 10 minutes per female (presented sequentially) to 2 days. However, most (36) studies did not report the experiment's duration and thus the relationship between time and hybrid matings could not be evaluated. A no-choice design was the most common experiment type (34), while tetrads (16) and dichotomous choice (12) were the next most common; only 5 presented females to males sequentially. Hybrid mating differed significantly among trial designs (Kruskal-Wallis χ^2 = 12.55, p = 0.0057); tetrad experiments had lower rates than all other types (Figure 4.11).

Table 4.2: Best models explaining variation in hybridization rate. Species pair is a random effect; all other explanatory variables are fixed effects. All models with ΔAICc <5 are shown.

Model	df	log(likelihood)	AICc	ΔAICc	weight
Species pair	3	25.8	-45.0	0.00	0.487
Species pair + F_{ST}	4	26.7	-44.4	0.58	0.364
Species pair + trial type	6	27.7	-41.5	3.50	0.084
Species pair + trial type + F_{ST}	7	28.4	-40.0	4.95	0.041

No-choice experiments varied substantially in the sex ratio: as many as 20 males or as few as 1 were placed with single females. While sex ratio appeared to have a strong effect on hybrid mating rate among no-choice trials (Figure 4.12), all of the trials with 1 female:20 males were from a single study (Jiggins et al. 2004) that tested courtship rates among subspecies of *H. melpomene*. Thus, the higher hybrid mating rate in this type of trial is likely a taxonomic artifact.

Hybridization rate declined slightly with sample size (slope = -0.002, r^2 = 0.05, p = 0.039; Figure 4.13). This may be partly due to a taxonomic bias towards larger samples for more isolated pairs, as F_{ST} was marginally significantly correlated with hybrid mating rate (slope = -0.25, r^2 = 0.05, p = 0.06; Figure 4.14), and significantly but weakly correlated with sample size (slope = -0.003, r^2 = 0.10, p = 0.013; Figure 4.15). Of perhaps greater interest is the fact that none of the experiments with the largest sample sizes had hybridization rates higher than 0.3.

Model selection found four models with ΔAICc <5 (Table 4.2). The best model included only the random effect of species pair, and the second-best model included species pair and F_{ST}.

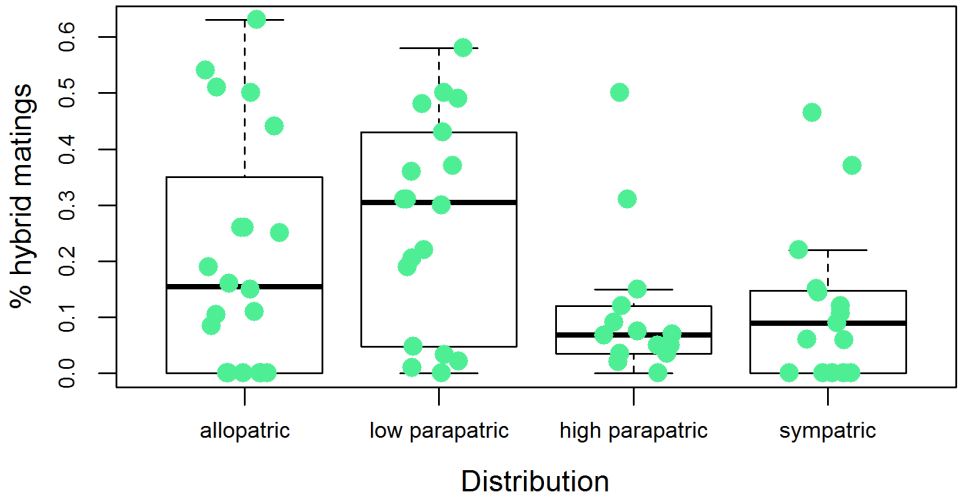

Figure 4.10: Hybrid mating rate versus species pair distribution.

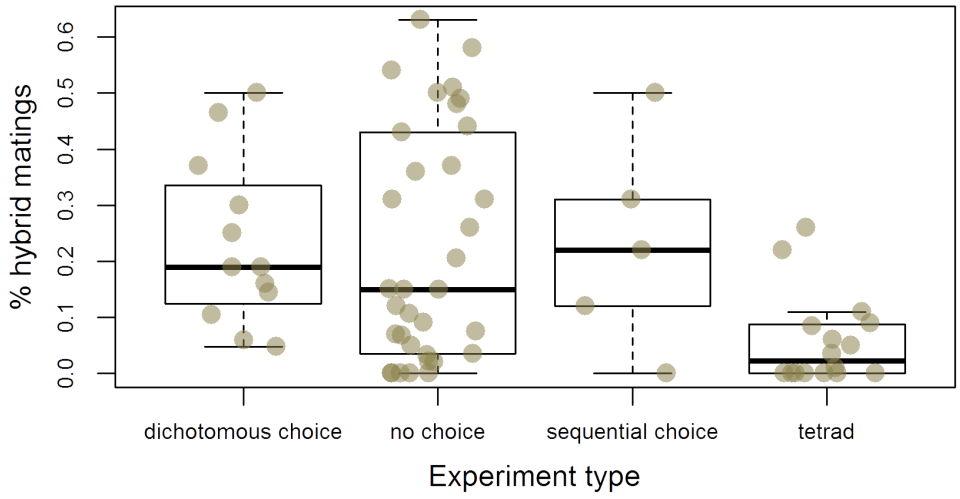

Figure 4.11: Hybrid mating rate versus experimental design.

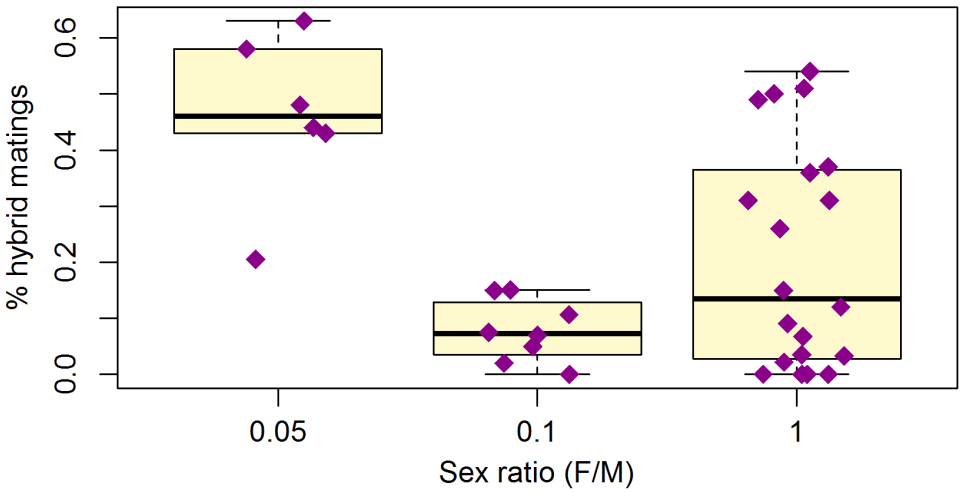

Figure 4.12: Hybrid mating rate versus sex ratio in no-choice experiments.

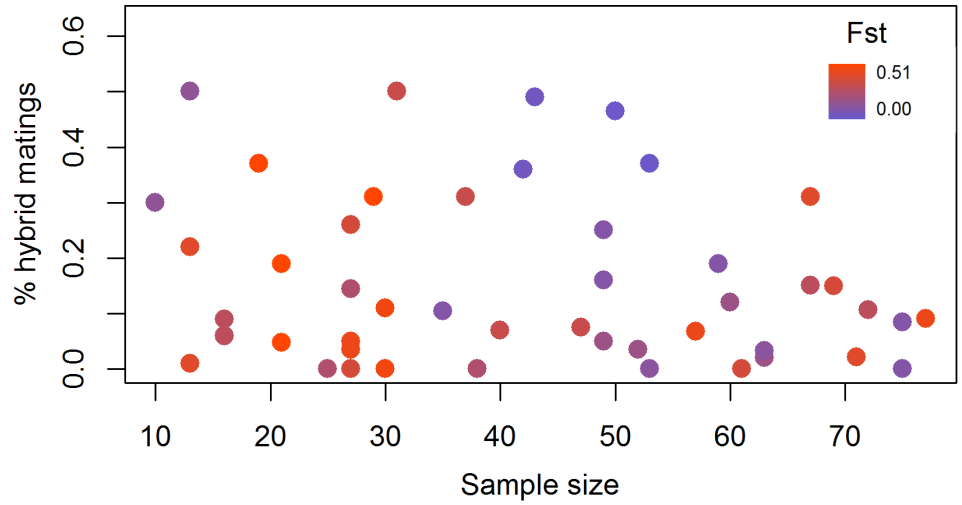

Figure 4.13: Percent of all matings that are interspecific/intersubspecific versus sample size. Shading of the points represents F_{ST} from low (blue) to high (orange).

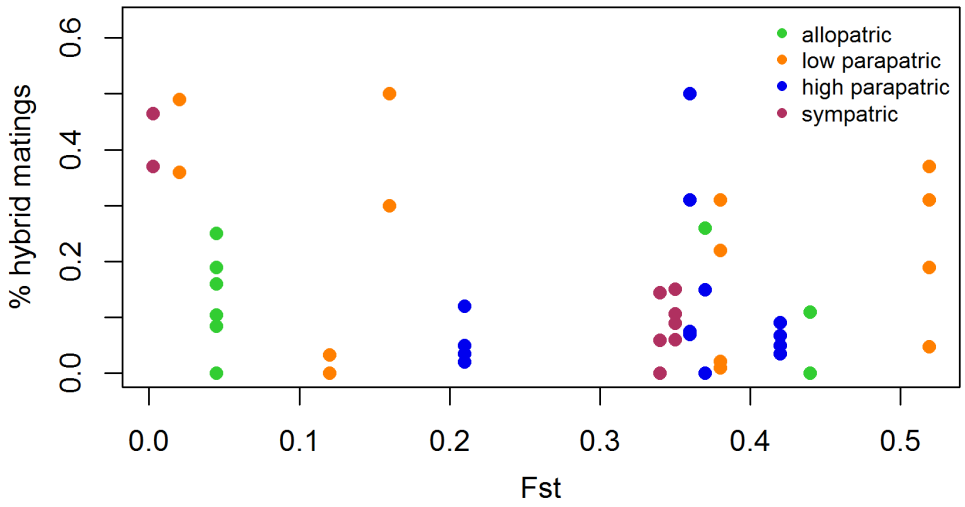

Figure 4.14: Hybrid mating rate versus F_{ST}. Points are coloured by species pair distribution type.

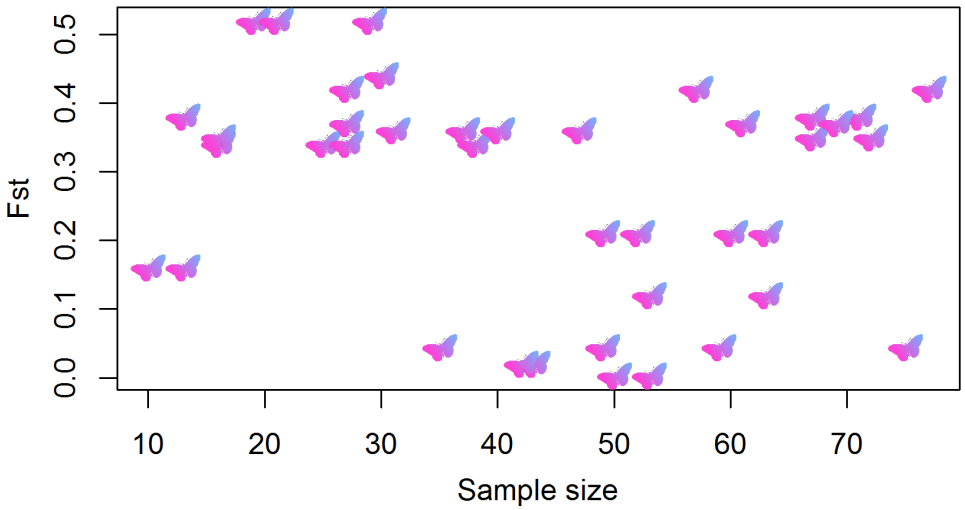

Figure 4.15: F_{ST} versus sample size.

Discussion

Mate choice between *Heliconius cydno* and *Heliconius pachinus*

Heliconius cydno and *H. pachinus* comprise one of the most-studied *Heliconius* species pairs. My results broadly agree with previous studies, which found strong assortative mating between the species. However, these results also differ from earlier findings in several notable ways. I examined F1 preference in depth because Kronforst et al. (2006b) found a difference between the preferences of F1s with *H. cydno* mothers and F1s with *H. pachinus* mothers, with the former slightly preferring *H. pachinus* females. In my study, F1 males preferred *H. pachinus* females regardless of the family of origin and the direction of the cross. In addition, F2 male preference differed from previous findings in that it was not associated with forewing or hindwing band colour. This genetic association between signal and preference has been documented in these and other *Heliconius* species (Kronforst et al. 2006b; Merrill et al. 2011b, 2019) and is predicted by theory on speciation with gene flow. Hindwing band colour has not been assessed in this way, but females' genotype at the hindwing band colour locus in *H. cydno* x *H. melpomene* backcrosses predicted their likelihood of mating (Merrill et al. 2011b).

Because my experiments differed from earlier studies in the populations of origin of the butterflies, the captive rearing environment, and the mate choice assessment method, there are many possible explanations for why these observations differed. Genetic and phenotypic differences in the source populations, founder effects in captivity, plastic reponses to the insectaries, the experimental protocol, and random noise could all explain differences in mate choice. My simulations of F2 preference suggest that my sample size was adequate to detect expected differences—though perhaps low sample size contributed to a lack of precision in estimates of those differences in the first place.

Mate choice across *Heliconius* and best practices for mate choice experiments

I explored mate choice across *Heliconius* in a meta-analysis both to examine the factors that affect premating isolation and to attempt to establish best practices in studying it. As expected, F_{ST} was negatively correlated with hybrid matings, meaning more genetically differentiated pairs were more

reproductively isolated. The correlation was extremely weak, however, perhaps because research has focused on pairs that hybridize in nature. Model selection suggested that an interaction between F_{ST} and species distribution overlap was not present. To increase the sample size in each range category, I binned allopatric with low parapatric and sympatric with high parapatric and reexamined this relationship, but found no significant effect of distribution overlap on preference. This may again be due to researchers' selection of (sub)species pairs, and it would be worth examining more pairs to determine whether the relationship between preference and genetic differentiation differs between sympatric and allopatric pairs, as in *Drosophila* (Coyne and Orr 1989, 1997). Previous findings of reproductive character displacement in some *Heliconius* pairs (Jiggins et al. 2001; Kronforst et al. 2007) hint that such a relationship may be present.

Trial type was the other factor included in the best explanatory models. While many researchers have considered the benefits and drawbacks of no-choice and dichotomous choice designs, the results of these trial types were not substantially different. The tetrad experiment type, in which a male and a female of each of two species are tested together, appears to be unique to *Heliconius* research, and consistently found lower hybrid matings, apparently not because of taxonomic bias. This may be because tetrads allow both male and female choice, whereas no-choice and dichotomous choice designs (especially those recording number of male courtships rather than achieved mating) may only allow male choice. However, no-choice experiments also allow both male and female choice, and no-choice experiments that used mating as the measure of preference had higher average preference than tetrad experiments. Furthermore, in the species/subspecies pairs that had data from both no-choice and tetrad experiments, preference was almost always higher in no-choice experiments. Thus, the ideal experimental design for estimating premating isolation in *Heliconius* warrants further study.

While sample size itself was not an important explanatory factor, the lack of high rates of hybrid matings in the experiments with the largest sample sizes is noteworthy. Experiments with small sample sizes may be more likely to overestimate rates of hybrid mating, because a single hybrid mating out of a small sample size changes the percentage disproportionately. While small sample sizes may be sufficient to establish whether a pair of species mates assortatively, they are unreliable when trying

to estimate reproductive isolation due to mate choice. Furthermore, many of these studies had highly unbalanced designs, with either much fewer conspecific or much fewer heterospecific trials, which both poses a problem for statistical testing and potentially hides the relationship between sample size and effect size.

Conclusions

I found assortative mating between *H. cydno galanthus* and *H. pachinus*, comparable to previous research. However, the preferences of F2 hybrid males was not consistent with earlier findings and with predictions that wing colour patterns and preference are genetically correlated, unlike in other *Heliconius* species. Across the genus, mate preference is affected primarily by species pair identity, with contributions from divergence (as measured by F_{ST}) and the type of behavioural assay used. The latter effect, as well as the lack of high hybridization rates in studies with large sample sizes, suggests that butterfly researchers should carefully consider experimental design when measuring mate choice.

REFERENCES

Abbott, R., D. Albach, S. Ansell, J. W. Arntzen, S. J. Baird, N. Bierne, J. Boughman, A. Brelsford, C. A. Buerkle, R. Buggs, et al., 2013. Hybridization and speciation. Journal of Evolutionary Biology 26:229–246.

Andrew, R. L. and L. H. Rieseberg, 2013. Divergence is focused on few genomic regions early in speciation: Incipient speciation of sunflower ecotypes. Evolution 67:2468–2482.

Barton, K., 2018. MuMIn: Multi-Model Inference. URL `https://CRAN.R-project.org/package=MuMIn`. R package version 1.42.1.

Barton, N., 1983. Multilocus clines. Evolution 37:454–471.

Barton, N. and B. O. Bengtsson, 1986. The barrier to genetic exchange between hybridising populations. Heredity 57:357–376.

Barton, N. H. and M. A. R. De Cara, 2009. The evolution of strong reproductive isolation. Evolution 63:1171–1190.

Beaumont, M. A., 2005. Adaptation and speciation: what can F(st) tell us? Trends in Ecology and Evolution 20:435–440.

Becquet, C. and M. Przeworski, 2009. Learning about modes of speciation by computational approaches. Evolution 63:2547–2562.

Bierne, N., 2010. The distinctive footprints of local hitchhiking in a varied environment and global hitchhiking in a subdivided population. Evolution 64:3254–3272.

Bierne, N., J. Welch, E. Loire, F. Bonhomme, and P. David, 2011. The coupling hypothesis: why genome scans may fail to map local adaptation genes. Molecular Ecology 20:2044–2072.

Bull, V., M. Beltrán, C. D. Jiggins, W. O. McMillan, E. Bermingham, and J. Mallet, 2006. Polyphyly and gene flow between non-sibling *Heliconius* species. BMC Biology 4:11.

Burri, R., A. Nater, T. Kawakami, C. F. Mugal, P. I. Olason, L. Smeds, et al., 2015. Linked selection and recombination rate variation drive the evolution of the genomic landscape of differentiation across the speciation continuum of *Ficedula* flycatchers. Genome Research 25:1656–1665.

Chouteau, M., V. Llaurens, F. Piron-Prunier, and M. Joron, 2017. Polymorphism at a mimicry supergene maintained by opposing frequency-dependent selection pressures. Proceedings of the National Academy of Sciences USA 114:8325–8329.

Costanzo, K. and A. Monteiro, 2007. The use of chemical and visual cues in female choice in the butterfly *Bicyclus anynana*. Proceedings of the Royal Society B — Biological Sciences 274:845–851.

Coyne, J. A. and H. A. Orr, 1989. Patterns of speciation in *Drosophila*. Evolution 43:362–381.

———, 1997. "Patterns of speciation in *Drosophila*" revisited. Evolution 51:295–303.

———, 2004. Speciation. Sinauer Associates, Sunderland, MA.

Crane, J., 1955. Imaginal behavior of a Trinidad butterfly, *Heliconius erato hydara* Hewitson, with special reference to the social use of color. Zoologica 40:167–196.

———, 1957. Imaginal behavior in butterflies of the family heliconiidae: changing social patterns and irrelevant actions. Zoologica 42:135–145.

Cruickshank, T. E. and M. W. Hahn, 2014. Reanalysis suggests that genomic islands of speciation are due to reduced diversity, not reduced gene flow. Molecular Ecology 23:3133–3157.

Darragh, K., S. Vanjari, F. Mann, M. F. Gonzalez-Rojas, C. R. Morrison, C. Salazar, C. Pardo-Diaz, R. M. Merrill, W. O. McMillan, S. Schulz, and C. D. Jiggins, 2017. Male sex pheromone components in *Heliconius* butterflies released by the androconia affect female choice. PeerJ 5:e3953.

Dukas, R. and A. Scott, 2015. Fruit fly courtship: The female perspective. Current Zoology 61:1008–1014.

Estrada, C. and C. D. Jiggins, 2008. Interspecific sexual attraction because of convergence in warning colouration: is there a conflict between natural and sexual selection in mimetic species? Journal of Evolutionary Biology 21:749–760.

Estrada, C., S. Schulz, S. Yildizhan, and L. E. Gilbert, 2011. Sexual selection drives the evolution of antiaphrodisiac pheromones in butterflies. Evolution 65:2843–2854.

Ewing, G. and J. Hermisson, 2010. Msms: a coalescent simulation program including recombination, demographic structure and selection at a single locus. Bioinformatics 26:2064–2065.

Excoffier, L. and N. Ray, 2008. Surfing during population expansions promotes genetic revolutions and structuration. Trends in Ecology and Evolution 23:347–351.

Feder, J. L., S. P. Egan, and P. Nosil, 2012. The genomics of speciation-with-gene-flow. Trends in Genetics 28:342–350.

Feder, J. L. and P. Nosil, 2010. The efficacy of divergence hitchhiking in generating genomic islands during ecological speciation. Evolution 64:1729–1747.

Feder, J. L., P. Nosil, A. C. Wacholder, S. P. Egan, S. H. Berlocher, and S. M. Flaxman, 2014. Genome-Wide Congealing and Rapid Transitions across the Speciation Continuum during Speciation with Gene Flow. Journal of Heredity 105:810–820.

Felsenstein, J., 1981. Skepticism Towards Santa Rosalia, or Why are There so Few Kinds of Animals? Evolution 35:124–138.

———, 1985. Phylogenies and the comparative method. The American Naturalist 125:1–15.

Finkbeiner, S. D., A. D. Briscoe, and R. D. Reed, 2014. Warning signals are seductive: relative contributions of color and pattern to predator avoidance and mate attraction in *Heliconius* butterflies. Evolution 68:3410–3420.

Finkbeiner, S. D., D. A. Fishman, D. Osorio, and A. D. Briscoe, 2017. Ultraviolet and yellow reflectance but not fluorescence is important for visual discrimination of conspecifics by *Heliconius erato*. Journal of Experimental Biology 220:1267–1276.

Flaxman, S. M., A. C. Wacholder, J. L. Feder, and P. Nosil, 2014. Theoretical models of the influence of genomic architecture on the dynamics of speciation. Moecularl Ecology 23:4074–4088.

Friberg, M., N. Vongvanich, A.-K. Borg-Karlson, D. J. Kemp, S. Merilaita, and C. Wiklund, 2008. Female mate choice determines reproductive isolation between sympatric butterflies. Behavioral Ecology and Sociobiology 62:873–886.

Gagnaire, P.-A., S. A. Pavey, E. Normandeau, and L. Bernatchez, 2013. The genetic architecture of reproductive isolation during speciation-with-gene-flow in lake whitefish species pairs assessed by RAD sequencing. Evolution 67:2483–2497.

Geritz, S. A., G. Mesze, J. A. Metz, et al., 1998. Evolutionarily singular strategies and the adaptive growth and branching of the evolutionary tree. Evolutionary Ecology 12:35–57.

Giraldo, N., C. Salazar, C. D. Jiggins, E. Bermingham, and M. Linares, 2008. Two sisters in the same dress: *Heliconius* cryptic species. BMC Evolutionary Biology 8:324.

Heliconius Genome Consortium, T., 2012. Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. Nature 487:94–98.

Hey, J. and R. Nielsen, 2004. Multilocus methods for estimating population sizes, migration rates and divergence time, with applications to the divergence of *Drosophila pseudoobscura* and *D. persimilis*. Genetics 167:747–760.

Hudson, R. R., 2002. Generating samples under a Wright-Fisher neutral model of genetic variation. Bioinformatics 18:337–338.

Hudson, R. R., M. Slatkin, and W. P. Maddison, 1992. Estimation of levels of gene flow from DNA sequence data. Genetics 132:583–589.

Ito, H. C. and U. Dieckmann, 2012. Evolutionary-branching lines and areas in bivariate trait spaces. Evoutionaryl Ecology Research 14:555–582.

Jennings, J. H., R. R. Snook, and A. Hoikkala, 2014. Reproductive isolation among allopatric *Drosophila montana* populations. Evolution 68:3095–3108.

Jiggins, C. D., 2016. The ecology and evolution of *Heliconius* butterflies. Oxford University Press.

Jiggins, C. D., C. Estrada, and A. Rodrigues, 2004. Mimicry and the evolution of premating isolation in *Heliconius melpomene* Linnaeus. Journal of Evolutionary Biology 17:680–691.

Jiggins, C. D., R. E. Naisbit, R. L. Coe, and J. Mallet, 2001. Reproductive isolation caused by colour pattern mimicry. Nature 411:302–305.

Kemp, D. J., 2007. Female butterflies prefer males bearing bright iridescent ornamentation. Proceedings of the Royal Society B — Biological Sciences 274:1043–1047.

Klein, A. L. and A. M. De Araújo, 2010. Courtship behavior of *Heliconius erato phyllis* (Lepidoptera, Nymphalidae) towards virgin and mated females: conflict between attraction and repulsion signals? Journal of Ethology 28:409–420.

Kronforst, M. R., 2008. Gene flow persists millions of years after speciation in *Heliconius* butterflies. BMC Evolutionary Biology 8:98.

Kronforst, M. R., M. E. B. Hansen, N. G. Crawford, J. R. Gallant, W. Zhang, R. J. Kulathinal, D. D. Kapan, and S. P. Mullen, 2013. Hybridization reveals the evolving genomic architecture of speciation. Cell Reports 5:1–12.

Kronforst, M. R. and R. Papa, 2015. The functional basis of wing patterning in *Heliconius* butterflies: the molecules behind mimicry. Genetics 200:1–19.

Kronforst, M. R., L. G. Young, L. M. Blume, and L. E. Gilbert, 2006a. Multilocus analyses of admixture and introgression among hybridizing *Heliconius* butterflies. Evolution 60:1254–1268.

Kronforst, M. R., L. G. Young, and L. E. Gilbert, 2007. Reinforcement of mate preference among hybridizing *Heliconius* butterflies. Journal of Evolutionary Biology 20:278–285.

Kronforst, M. R., L. G. Young, D. D. Kapan, C. McNeely, R. J. O'Neill, and L. E. Gilbert, 2006b. Linkage of butterfly mate preference and wing color preference cue at the genomic location of wingless. Proceedings of the National Academy of Sciences USA 103:6575–6580.

Mallet, J., M. Beltrán, W. Neukirchen, and M. Linares, 2007. Natural hybridization in heliconiine butterflies: the species boundary as a continuum. BMC Evolutionary Biology 7:28.

Martin, S. H., K. K. Dasmahapatra, N. J. Nadeau, C. Salazar, J. R. Walters, F. Simpson, et al., 2013. Genome-wide evidence for speciation with gene flow in heliconius butterflies. Genome Res 23:1817–1828.

Mavárez, J., C. A. Salazar, E. Bermingham, C. Salcedo, C. D. Jiggins, and M. Linares, 2006. Speciation by hybridization in *Heliconius* butterflies. Nature 441:868–871.

McMillan, W. O., C. D. Jiggins, and J. Mallet, 1997. What initiates speciation in passion-vine butterflies? Proceedings of the National Academy of Sciences 94:8628–8633.

McNiven, V. T. and A. J. Moehring, 2013. Identification of genetically linked female preference and male trait. Evolution 67:2155–2165.

McPeek, M. A., L. B. Symes, D. M. Zong, and C. L. McPeek, 2011. Species recognition and patterns of population variation in the reproductive structures of a damselfly genus. Evolution 65:419–428.

Mérot, C., B. Frérot, E. Leppik, and M. Joron, 2015. Beyond magic traits: multimodal mating cues in *Heliconius* butterflies. Evolution 69:2891–2904.

Mérot, C., C. Salazar, R. M. Merrill, C. D. Jiggins, and M. Joron, 2017. What shapes the continuum of reproductive isolation? Lessons from *Heliconius* butterflies. Proceedings of the Royal Society B: Biological Sciences 284:20170335.

Merrill, R., K. Dasmahapatra, J. Davey, D. Dell'Aglio, J. Hanly, B. Huber, C. Jiggins, M. Joron, K. Kozak, V. Llaurens, et al., 2015. The diversification of *Heliconius* butterflies: what have we learned in 150 years? Journal of Evolutionary Biology 28:1417–1438.

Merrill, R. M., A. Chia, and N. J. Nadeau, 2014. Divergent warning patterns contribute to assortative mating between incipient *Heliconius* species. Ecology and Evolution 4:911–917.

Merrill, R. M., Z. Gompert, L. M. Dembeck, M. R. Kronforst, W. O. McMillan, and C. D. Jiggins, 2011a. Mate preference across the speciation continuum in a clade of mimetic butterflies. Evolution 65:1489–500.

Merrill, R. M., P. Rastas, S. H. Martin, M. C. Melo, S. Barker, J. Davey, W. O. McMillan, and C. D. Jiggins, 2019. Genetic dissection of assortative mating behavior. PLoS Biology 17:e2005902.

Merrill, R. M., B. V. Schooten, J. A. Scott, and C. D. Jiggins, 2011b. Pervasive genetic associations between traits causing reproductive isolation in *Heliconius* butterflies. Proceedings of the Royal Society B — Biological Sciences 278:511–518.

Merrill, R. M., R. W. Wallbank, V. Bull, P. C. Salazar, J. Mallet, M. Stevens, and C. D. Jiggins, 2012. Disruptive ecological selection on a mating cue. Proceedings of the Royal Society B — Biological Sciences 279:4907–4913.

Morehouse, N. I. and R. L. Rutowski, 2010. In the eyes of the beholders: female choice and avian predation risk associated with an exaggerated male butterfly color. The American Naturalist 176:768–784.

Munoz, A. G., C. Salazar, J. Castano, C. D. Jiggins, and M. Linares, 2010. Multiple sources of reproductive isolation in a bimodal butterfly hybrid zone. Journal of Evolutionary Biology 23:1312–1320.

Nadeau, N. J., S. H. Martin, K. M. Kozak, C. Salazar, K. K. Dasmahapatra, J. W. Davey, S. W. Baxter, M. L. Blaxter, J. Mallet, and C. D. Jiggins, 2013. Genome-wide patterns of divergence and gene flow across a butterfly radiation. Molecular Ecology 22:814–826.

Nadeau, N. J., C. Pardo-Diaz, A. Whibley, M. A. Supple, S. V. Saenko, R. W. Wallbank, G. C. Wu, L. Maroja, L. Ferguson, J. J. Hanly, et al., 2016. The gene *cortex* controls mimicry and crypsis in butterflies and moths. Nature 534:106–110.

Nadeau, N. J., A. Whibley, R. T. Jones, J. W. Davey, K. K. Dasmahapatra, S. W. Baxter, et al., 2012. Genomic islands of divergence in hybridizing *Heliconius* butterflies identified by large-scale targeted sequencing. Philosophical Transactions of the Royal Society B 367:343–353.

Naisbit, R. E., C. D. Jiggins, M. Linares, C. Salazar, and J. Mallet, 2002. Hybrid sterility, Haldane's rule and speciation in *Heliconius cydno* and *H. melpomene*. Genetics 161:1517–1526.

Naisbit, R. E., C. D. Jiggins, and J. Mallet, 2001. Disruptive sexual selection against hybrids contributes to speciation between *Heliconius cydno* and *Heliconius melpomene*. Proceedings of the Royal Society of London. Series B: Biological Sciences 268:1849–1854.

Nei, M., 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Nielsen, R. and J. Wakeley, 2001. Distinguishing migration from isolation: a Markov chain Monte Carlo approach. Genetics 158:885–896.

Noor, M. A., 1995. Speciation driven by natural selection in *Drosophila*. Nature 375:674–675.

Noor, M. A. F., 1999. Reinforcement and other consequences of sympatry. Heredity 83:503–508.

Nosil, P., B. J. Crespi, and C. P. Sandoval, 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proceedings of the Royal Society B — Biological Sciences 270:1911–1918.

Nosil, P. and J. L. Feder, 2012. Genomic divergence during speciation: causes and consequences. Philosophical Transactions of the Royal Society B 367:332–342.

Nosil, P., D. J. Funk, and D. Ortiz-Barrientos, 2009. Divergent selection and heterogeneous genomic divergence. Molecular Ecology 18:375–402.

Orr, H. A., 1995. The population genetics of speciation: the evolution of hybrid incompatibilities. Genetics 139:1805–1813.

Ortiz-Barrientos, D., A. Grealy, and P. Nosil, 2009. The genetics and ecology of reinforcement: Implications for the evolution of prezygotic isolation in sympatry and beyond. Annals of the New York Academy of Science 1168:156–182.

Pfeifer, B., U. Wittelsbürger, S. E. Ramos-Onsins, and M. J. Lercher, 2014. PopGenome: An efficient swiss army knife for population genomic analyses in R. Molecular Biology and Evolution 31:1929–1936.

Pfennig, K. S. and A. M. Rice, 2014. Reinforcement generates reproductive isolation between neighbouring conspecific populations of spadefoot toads. Proceedings of the Royal Society B — Biological Sciences 281:20140949.

Powell, T. H., D. H. Cha, C. E. Linn, and J. L. Feder, 2012. On the scent of standing variation for speciation: behavioral evidence for native sympatric host races of *Rhagoletis pomonella* (Diptera: Tephritidae) in the southern United States. Evolution 66:2739–2756.

R Core Team, 2013. R: A language and environment for statistical computing. URL `http://www.r-project.org/`.

Rambaut, A. and N. C. Grassly, 1997. Seq-Gen: an application for the Monte Carlo simulation of DNA sequence evolution along phylogenetic trees. Computer Applications in the Biosciences 13:235–238.

Ramsey, J., H. Bradshaw, and D. W. Schemske, 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57:1520–1534.

Renaut, S., C. J. Grassa, S. Yeaman, B. T. Moyers, Z. Lai, N. C. Kane, et al., 2013. Genomic islands of divergence are not affected by geography of speciation in sunflowers. Nature Communications 4:1827.

Renaut, S., N. Maillet, E. Normandeau, C. Sauvage, N. Derome, S. M. Rogers, and L. Bernatchez, 2012. Genome-wide patterns of divergence during speciation: the lake whitefish case study. Philosophical Transactions of the Royal Society B 367:354–363.

Renaut, S., G. L. Owens, and L. H. Rieseberg, 2014. Shared selective pressure and local genomic landscape lead to repeatable patterns of genomic divergence in sunflowers. Molecular Ecology 23:311–324.

Rieseberg, L. H. and J. M. Burke, 2001. A genic view of species integration. Journal of Evolutionary Biology 14:883–886.

Robertson, K. A. and A. Monteiro, 2005. Female *Bicyclus anynana* butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. Proceedings of the Royal Society B — Biological Sciences 272:1541–1546.

Roesti, M., S. Gavrilets, A. P. Hendry, W. Salzburger, and D. Berner, 2014. The genomic signature of parallel adaptation from shared genetic variation. Molecular Ecology 23:3944–3956.

Roesti, M., A. P. Hendry, W. Salzburger, and D. Berner, 2012. Genome divergence during evolutionary diversification as revealed in replicate lake-stream stickleback population pairs. Molecular Ecology 21:2852–2862.

Rosser, N., A. B. Phillimore, B. Huertas, K. R. Willmott, and J. Mallet, 2012. Testing historical explanations for gradients in species richness in heliconiine butterflies of tropical America. Biological Journal of the Linnean Society 105:479–497.

Rutowski, R. L. and P. K. Rajyaguru, 2013. Male-specific iridescent coloration in the pipevine swallowtail (*Battus philenor*) is used in mate choice by females but not sexual discrimination by males. Journal of Insect Behaviour 26:200–211.

Sánchez, A. P., C. Pardo-Diaz, J. Enciso-Romero, A. Muñoz, C. D. Jiggins, C. Salazar, and M. Linares, 2015. An introgressed wing pattern acts as a mating cue. Evolution 69:1619–1629.

Schemske, D. W., 2000. Understanding the origin of species. Evolution 54:1069–1073.

Schluter, D., 2009. Evidence for ecological speciation and its alternative. Nature 323:737–741.

Schulz, S., S. Yildizhan, K. Stritzke, C. Estrada, and L. E. Gilbert, 2007. Macrolides from the scent glands of the tropical butterflies *Heliconius cydno* and *Heliconius pachinus*. Organic and Biomolecular Chemistry 5:3434–3441.

Seehausen, O., R. K. Butlin, I. Keller, C. E. Wagner, J. W. Boughman, P. A. Hohenlohe, C. L. Peichel, G.-P. Saetre, C. Bank, A. Brännström, A. Brelsford, C. S. Clarkson, F. Eroukhmanoff, J. L. Feder, M. C. Fischer, A. D. Foote, P. Franchini, C. D. Jiggins, F. C. Jones, A. K. Lindholm, K. Lucek, M. E. Maan, D. A. Marques, S. H. Martin, B. Matthews, J. I. Meier, M. Möst, M. W. Nachman, E. Nonaka, D. J. Rennison, J. Schwarzer, E. T. Watson, A. M. Westram, and A. Widmer, 2014. Genomics and the origin of species. Nature Reviews Genetics 15:176–192.

Servedio, M. R., 2009. The role of linkage disequilibrium in the evolution of premating isolation. Heredity 102:51–56.

Silberglied, R. E. and O. R. Taylor, 1978. Ultraviolet reflection and its behavioral role in the courtship of the sulfur butterflies *Colias eurytheme* and *C. philodice* (Lepidoptera, Pieridae). Behavioral Ecology and Sociobiology 3:203–243.

Soria-Carrasco, V., Z. Gompert, A. A. Comeault, T. E. Farkas, T. L. Parchman, J. S. Johnston, C. A. Buerkle, J. L. Feder, J. Bast, T. Schwander, S. P. Egan, B. J. Crespi, and P. Nosil, 2014. Stick insect genomes reveal natural selection's role in parallel speciation. Science 344:738–742.

Southcott, L. and M. R. Kronforst, 2018. Data from: Female mate choice is a reproductive isolating barrier in heliconius butterflies. URL `https://doi.org/10.5061/dryad.f57g6h5`.

Strasburg, J. L., N. A. Sherman, K. M. Wright, L. C. Moyle, J. H. Willis, and L. H. Rieseberg, 2012. What can patterns of differentiation across plant genomes tell us about adaptation and speciation? Philosophical Transactions of the Royal Society B 367:364–373.

Turner, T. L., M. W. Hahn, and S. V. Nuzhdin, 2005. Genomic islands of speciation in *Anopheles gambiae*. PLOS Biology 3:e285.

Twyford, A. D. and R. A. Ennos, 2012. Next-generation hybridization and introgression. Heredity 108:179–189.

Via, S. and J. West, 2008. The genetic mosaic suggests a new role for hitchhiking in ecological speciation. Molecular Ecology 17:4334–4345.

Walters, J. R., C. Stafford, T. J. Hardcastle, and C. D. Jiggins, 2012. Evaluating female remating rates in light of spermatophore degradation in *Heliconius* butterflies: pupal-mating monandry versus adult-mating polyandry. Ecological Entomology 37:257–268.

Westerman, E. L., N. W. VanKuren, D. Massardo, A. Tenger-Trolander, W. Zhang, R. I. Hill, M. Perry, E. Bayala, K. Barr, N. Chamberlain, et al., 2018. *Aristaless* controls butterfly wing color variation used in mimicry and mate choice. Current Biology 28:3469–3474.

Wiernasz, D. C. and J. G. Kingsolver, 1992. Wing melanin pattern mediates species recognition in *Pieris occidentalis*. Animal Behaviour 43:89–94.

Wiley, C., C. K. Ellison, and K. L. Shaw, 2011. Widespread genetic linkage of mating signals and preferences in the Hawaiian cricket *Laupala*. Proceedings of the Royal Society B — Biological Sciences P. rspb20111740.

Wiley, C. and K. L. Shaw, 2010. Multiple genetic linkages between female preference and male signal in rapidly speciating Hawaiian crickets. Evolution 64:2238–2245.

Wolf, J. B. and H. Ellegren, 2016. Making sense of genomic islands of differentiation in light of speciation. Nature Reviews Genetics .

Wu, C.-I., 2001. The genic view of the process of speciation. Journal of Evolutionary Biology 14:851–865.

Wu, G. C., M. Joron, and C. D. Jiggins, 2010. Signatures of selection in loci governing major colour patterns in *Heliconius* butterflies and related species. BMC Evolutionary Biology 10:368.

Wutzler, T., 2018. logitnorm: Functions for the Logitnormal Distribution. URL `https://CRAN.R-project.org/package=logitnorm`. R package version 0.8.37.

Zhang, W., K. K. Dasmahapatra, J. Mallet, G. R. Moreira, and M. R. Kronforst, 2016. Genome-wide introgression among distantly related *Heliconius* butterfly species. Genome Biology 17:25.

APPENDIX A

SUPPLEMENTAL MATERIAL FOR CHAPTER 2

Simulation Parameters

The following are the input codes for ms (or msms) and Seq-Gen for each simulation scenario with an effective population size of 10^6.

No selection

No gene flow

```
./ms 160 1 -T -r 4000 100000 -I 16 10 10 10 10 10 10 10 10 10 10
    10 10 10 10 10 10 -ej .5 1 2 -en .5 2 .5 -ej 1 3 4 -en 1 4 .5
    -ej 1.5 2 4 -en 1.5 4 .5 -ej 1.5 5 6 -en 1.5 6 .5 -ej 2 4 6 -
    en 2 6 .5 -ej 2 7 8 -en 2 8 .5 -ej 2.5 6 8 -en 2.5 8 .5 -ej
    2.5 9 10 -en 2.5 10 .5 -ej 3 8 10 -en 3 10 .5 -ej 3 11 12 -en
    3 12 .5 -ej 3.5 10 12 -en 3.5 12 .5 -ej 3.5 13 14 -en 3.5 14
    .5 -ej 4 12 14 -en 4 14 .5 -ej 4 15 16 -en 4 16 .5 -ej 4.5 14
    16 -en 4.5 16 .5
```

```
./Seq-Gen.v1.3.3/source/seq-gen -mHKY -l 100000 -s .02 -p 483605
```

Early gene flow

```
./ms 160 1 -T -r 4000 100000 -I 16 10 10 10 10 10 10 10 10 10 10
    10 10 10 10 10 10 -m 2 1 10.0 -ej .5 1 2 -en .5 2 .5 -em .5 4
    3 10.0 -ej 1 3 4 -en 1 4 .5 -em 1 6 5 10.0 -ej 1.5 2 4 -en 1.5
     4 .5 -ej 1.5 5 6 -en 1.5 6 .5 -em 1.5 8 7 10.0 -ej 2 4 6 -en
    2 6 .5 -ej 2 7 8 -en 2 8 .5 -em 2 10 9 10.0 -ej 2.5 6 8 -en
```

```
2.5 8 .5 -ej 2.5 9 10 -en 2.5 10 .5 -em 2.5 12 11 10.0 -ej 3 8
 10 -en 3 10 .5 -ej 3 11 12 -en 3 12 .5 -em 3 14 13 10.0 -ej
3.5 10 12 -en 3.5 12 .5 -ej 3.5 13 14 -en 3.5 14 .5 -em 3.5 16
 15 10.0 -ej 4 12 14 -en 4 14 .5 -ej 4 15 16 -en 4 16 .5 -ej
4.5 14 16 -en 4.5 16 .5

./Seq-Gen.v1.3.3/source/seq-gen -mHKY -l 100000 -s .02 -p 483605
```

Recent gene flow

```
./ms 160 1 -T -r 4000 100000 -I 16 10 10 10 10 10 10 10 10 10 10
   10 10 10 10 10 10 -m 2 1 1 -m 4 3 1 -m 6 5 1 -m 8 7 1 -m 10 9
   1 -m 12 11 1 -m 14 13 1 -m 16 15 1 -eM .5 0 -ej .5 1 2 -en .5
   2 .5 -ej 1 3 4 -en 1 4 .5 -ej 1.5 2 4 -en 1.5 4 .5 -ej 1.5 5 6
    -en 1.5 6 .5 -ej 2 4 6 -en 2 6 .5 -ej 2 7 8 -en 2 8 .5 -ej
   2.5 6 8 -en 2.5 8 .5 -ej 2.5 9 10 -en 2.5 10 .5 -ej 3 8 10 -en
    3 10 .5 -ej 3 11 12 -en 3 12 .5 -ej 3.5 10 12 -en 3.5 12 .5 -
   ej 3.5 13 14 -en 3.5 14 .5 -ej 4 12 14 -en 4 14 .5 -ej 4 15 16
    -en 4 16 .5 -ej 4.5 14 16 -en 4.5 16 .5

./Seq-Gen.v1.3.3/source/seq-gen -mHKY -l 100000 -s .02 -p 483605
```

The migration parameter (4Nm, the third value after the -m switches) was set to 0.001, 0.01, 0.1, 1, or 10 for simulations with a population size of 10^6. For population sizes of 10^5, migration parameters were 0.0001, 0.01, and 1; for N = 10^4, they were 0.00001, 0.001, and 0.1. These migration parameters have the same migration proportions as 4Nm = 0.001, 0.1, and 10 for N = 10^6.

Divergent selection

No gene flow

```
./msms 160 1 -T -r 4000 100000 -N 1000000 -I 16 10 10 10 10 10 10
    10 10 10 10 10 10 10 10 10 10 -ej .5 1 2 -en .5 2 .5 -ej 1 3
    4 -en 1 4 .5 -ej 1.5 2 4 -en 1.5 4 .5 -ej 1.5 5 6 -en 1.5 6 .5
    -ej 2 4 6 -en 2 6 .5 -ej 2 7 8 -en 2 8 .5 -ej 2.5 6 8 -en 2.5
    8 .5 -ej 2.5 9 10 -en 2.5 10 .5 -ej 3 8 10 -en 3 10 .5 -ej 3
    11 12 -en 3 12 .5 -ej 3.5 10 12 -en 3.5 12 .5 -ej 3.5 13 14 -
    en 3.5 14 .5 -ej 4 12 14 -en 4 14 .5 -ej 4 15 16 -en 4 16 .5 -
    ej 4.5 14 16 -en 4.5 16 .5 -SI 2 16 .5 .5 .5 .5 .5 .5 .5 .5 .5
    .5 .5 .5 .5 .5 .5 .5 -Sc 0 16 0 100000 200000 -Sc 0 15 200000
    100000 0 -Sc 0 14 0 100000 200000 -Sc 0 13 200000 100000 0 -
    Sc 0 12 0 100000 200000 -Sc 0 11 200000 100000 0 -Sc 0 10 0
    100000 200000 -Sc 0 9 200000 100000 0 -Sc 0 8 0 100000 200000
    -Sc 0 7 200000 100000 0 -Sc 0 6 0 100000 200000 -Sc 0 5 200000
    100000 0 -Sc 0 4 0 100000 200000 -Sc 0 3 200000 100000 0 -Sc
    0 2 0 100000 200000 -Sc 0 1 200000 100000 0 -Sc .5 2 0 0 0 -Sc
    1 4 0 0 0 -Sc 1.5 6 0 0 0 -Sc 2 8 0 0 0 -Sc 2.5 10 0 0 0 -Sc
    3 12 0 0 0 -Sc 3.5 14 0 0 0 -Sc 4 16 0 0 0
```

```
./Seq-Gen.v1.3.3/source/seq-gen -mHKY -l 100000 -s .02 -p 483605
```

Early gene flow

```
./msms 160 1 -T -r 4000 100000 -N 1000000 -I 16 10 10 10 10 10 10
    10 10 10 10 10 10 10 10 10 10 -m 2 1 10.0 -ej .5 1 2 -en .5 2
    .5 -em .5 4 3 10.0 -ej 1 3 4 -en 1 4 .5 -em 1 6 5 10.0 -ej
    1.5 2 4 -en 1.5 4 .5 -ej 1.5 5 6 -en 1.5 6 .5 -em 1.5 8 7 10.0
    -ej 2 4 6 -en 2 6 .5 -ej 2 7 8 -en 2 8 .5 -em 2 10 9 10.0 -ej
    2.5 6 8 -en 2.5 8 .5 -ej 2.5 9 10 -en 2.5 10 .5 -em 2.5 12 11
```

```
10.0 -ej 3 8 10 -en 3 10 .5 -ej 3 11 12 -en 3 12 .5 -em 3 14
13 10.0 -ej 3.5 10 12 -en 3.5 12 .5 -ej 3.5 13 14 -en 3.5 14
.5 -em 3.5 16 15 10.0 -ej 4 12 14 -en 4 14 .5 -ej 4 15 16 -en
4 16 .5 -ej 4.5 14 16 -en 4.5 16 .5 -SI 2 16 .5 .5 .5 .5 .5 .5
.5 .5 .5 .5 .5 .5 .5 .5 .5 .5 -Sc 0 16 0 100000 200000 -Sc 0
15 200000 100000 0 -Sc 0 14 0 100000 200000 -Sc 0 13 200000
100000 0 -Sc 0 12 0 100000 200000 -Sc 0 11 200000 100000 0 -Sc
0 10 0 100000 200000 -Sc 0 9 200000 100000 0 -Sc 0 8 0 100000
200000 -Sc 0 7 200000 100000 0 -Sc 0 6 0 100000 200000 -Sc 0
5 200000 100000 0 -Sc 0 4 0 100000 200000 -Sc 0 3 200000
100000 0 -Sc 0 2 0 100000 200000 -Sc 0 1 200000 100000 0 -Sc
.5 2 0 0 0 -Sc 1 4 0 0 0 -Sc 1.5 6 0 0 0 -Sc 2 8 0 0 0 -Sc 2.5
10 0 0 0 -Sc 3 12 0 0 0 -Sc 3.5 14 0 0 0 -Sc 4 16 0 0 0
```

```
./Seq-Gen.v1.3.3/source/seq-gen -mHKY -l 100000 -s .02 -p 483605
```

Recent gene flow

```
./msms 160 1 -T -r 4000 100000 -N 1000000 -I 16 10 10 10 10 10 10
10 10 10 10 10 10 10 10 10 10 -m 2 1 10.0 -m 4 3 10.0 -m 6 5
10.0 -m 8 7 10.0 -m 10 9 10.0 -m 12 11 10.0 -m 14 13 10.0 -m
16 15 10.0 -eM .5 0 -ej .5 1 2 -en .5 2 .5 -ej 1 3 4 -en 1 4
.5 -ej 1.5 2 4 -en 1.5 4 .5 -ej 1.5 5 6 -en 1.5 6 .5 -ej 2 4 6
-en 2 6 .5 -ej 2 7 8 -en 2 8 .5 -ej 2.5 6 8 -en 2.5 8 .5 -ej
2.5 9 10 -en 2.5 10 .5 -ej 3 8 10 -en 3 10 .5 -ej 3 11 12 -en
3 12 .5 -ej 3.5 10 12 -en 3.5 12 .5 -ej 3.5 13 14 -en 3.5 14
.5 -ej 4 12 14 -en 4 14 .5 -ej 4 15 16 -en 4 16 .5 -ej 4.5 14
16 -en 4.5 16 .5 -SI 2 16 .5 .5 .5 .5 .5 .5 .5 .5 .5 .5 .5
```

```
.5 .5 .5 .5 –Sc 0 16 0 100000 200000 –Sc 0 15 200000 100000 0
–Sc 0 14 0 100000 200000 –Sc 0 13 200000 100000 0 –Sc 0 12 0
100000 200000 –Sc 0 11 200000 100000 0 –Sc 0 10 0 100000
200000 –Sc 0 9 200000 100000 0 –Sc 0 8 0 100000 200000 –Sc 0 7
 200000 100000 0 –Sc 0 6 0 100000 200000 –Sc 0 5 200000 100000
 0 –Sc 0 4 0 100000 200000 –Sc 0 3 200000 100000 0 –Sc 0 2 0
100000 200000 –Sc 0 1 200000 100000 0 –Sc .5 2 0 0 0 –Sc 1 4 0
 0 0 –Sc 1.5 6 0 0 0 –Sc 2 8 0 0 0 –Sc 2.5 10 0 0 0 –Sc 3 12 0
 0 0 –Sc 3.5 14 0 0 0 –Sc 4 16 0 0 0
```

```
./Seq–Gen.v1.3.3/source/seq–gen –mHKY –l 100000 –s .02 –p 483605
```

Supplemental Results

Bidirectional gene flow

We simulated bidirectional gene flow without selection between sister species in the first 2N generations after divergence and the most recent 2N generations at effective migration parameters of 0.001, 0.01, 0.1, 1, and 10. As for unidirectional gene flow, there was a faster than linear increase in the size of the divergent genome with all levels of early gene flow and with recent gene flow of up to 4Nm = 0.01 (Figure A1). Higher recent gene flow levels slowed (4Nm = 1) or eliminated (4Nm = 10) this increase.

Threshold value

To ensure that the observed shape of growth of the highly divergent portion of the genome was not an artifact of the choice of threshold value for designating windows as F_{ST} outliers, we compared 80th, 95th, and 99th percentile thresholds and the 95th percentile with all intervening windows above the 75th percentile as described in the Methods. All simulations have $N = 10^6$. Regardless of threshold

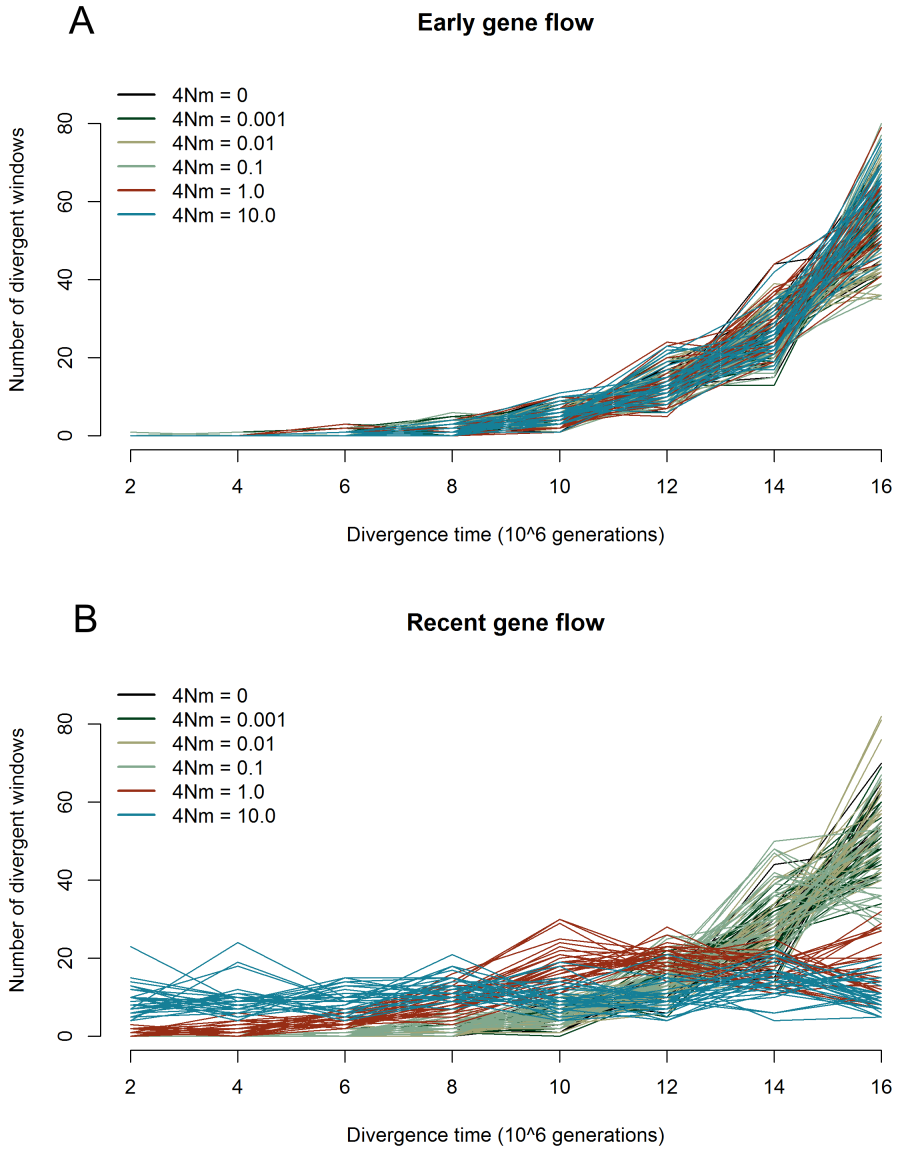

Figure A1: A: Divergent genome size (number of outlier windows) increases faster than linearly with divergence time when no gene flow or various levels of bidirectional early gene flow occurs. B: The highest levels of bidirectional recent gene flow homogenize the genome and prevent this increase. Each line represents a single simulation of eight between-species comparisons.

level, recent gene flow (4Nm = 10) always produced a flat line, while the no gene flow scenario showed a faster than linear increase (Figure A2).

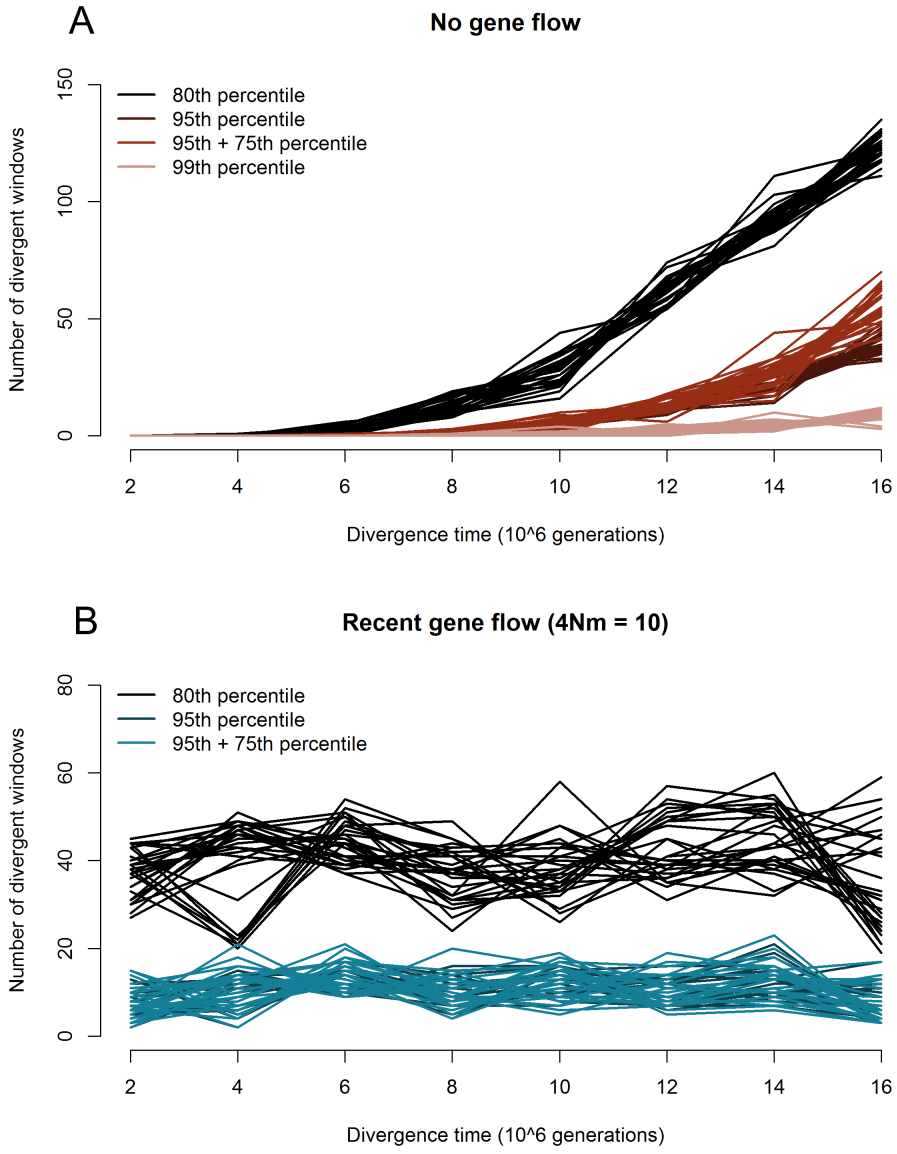

Figure A2: A: Changing the F_{ST} outlier threshold changes the steepness but not the shape (faster than linear) of the relationship between outlier size and divergence time in the no gene flow scenario. B: Changing the F_{ST} outlier threshold changes the number of windows classified as outliers but still shows a lack of growth in number of outliers with divergence time in the recent gene flow (unidirectional, 4Nm = 10) scenario.

Number of fixed differences

We computed the numbers of fixed differences between sister species in each simulation to see how they change with divergence time and with gene flow/selection scenario. We expect this statistic to increase linearly with increasing time since divergence without gene flow or selection. This was the

case for all early gene flow scenarios. The rate of increase in fixed differences slowed with higher recent gene flow without selection, and underwent a nonlinear increase followed by slower increase in high recent gene flow scenarios with selecion (Figure A3).

F_{ST} versus divergence time

We compared both mean (over 500 bp windows) and global F_{ST} of simulated 100 kbp genomes versus divergence time between sister species (Figure A4). When no gene flow was present, F_{ST} increased at a diminishing rate, reaching a plateau at higher divergence times. High (4Nm = 10) recent gene flow produced no change in either mean or global F_{ST}. Predicted F_{ST} for the no migration scenario is shown in Figure A4. These values were obtained using the formula $F_{ST} = 1 - E(t_s)/E(t_d)$, where $E(t_s)$ is the expected time to coalescence for two alleles sampled from the same population and $E(t_d)$ is the expected coalescence time for two alleles sampled from different populations. From coalescent theory, $E(t_s)$ = 2N when there is no migration and N is the population size. $E(t_d)$ is the sum of the time backwards from the present to population splitting (4NT, where T is any positive number) and the expected time to coalescence before population splitting (2N, since we simulated populations that retained the same population size after splitting rather than being halved). Thus, predicted $F_{ST} = 1 - \frac{1}{2T+1}$.

Figures A5 and A6 show the change in distribution of window F_{ST} values with increasing divergence time in the no selection no gene flow and recent gene flow (4Nm = 10) scenarios respectively for a representative run of each simulation. They clearly show a rightward shift in F_{ST} over time when there is no migration, but show no change when high gene flow is simulated. Figures A7 and A8 likewise show the change in distribution of F_{ST} in simulations with divergent selection and no gene flow or high recent gene flow (4Nm = 10). The former resembles the same scenario without selection (Figure A5), while the latter shows a transition to higher F_{ST} values between 6N and 8N generations. Figures A9 zooms in on the extreme right tails of the distributions in Figure A5 to show the exponential increase in the number of outlier windows above the 95th percentile threshold.

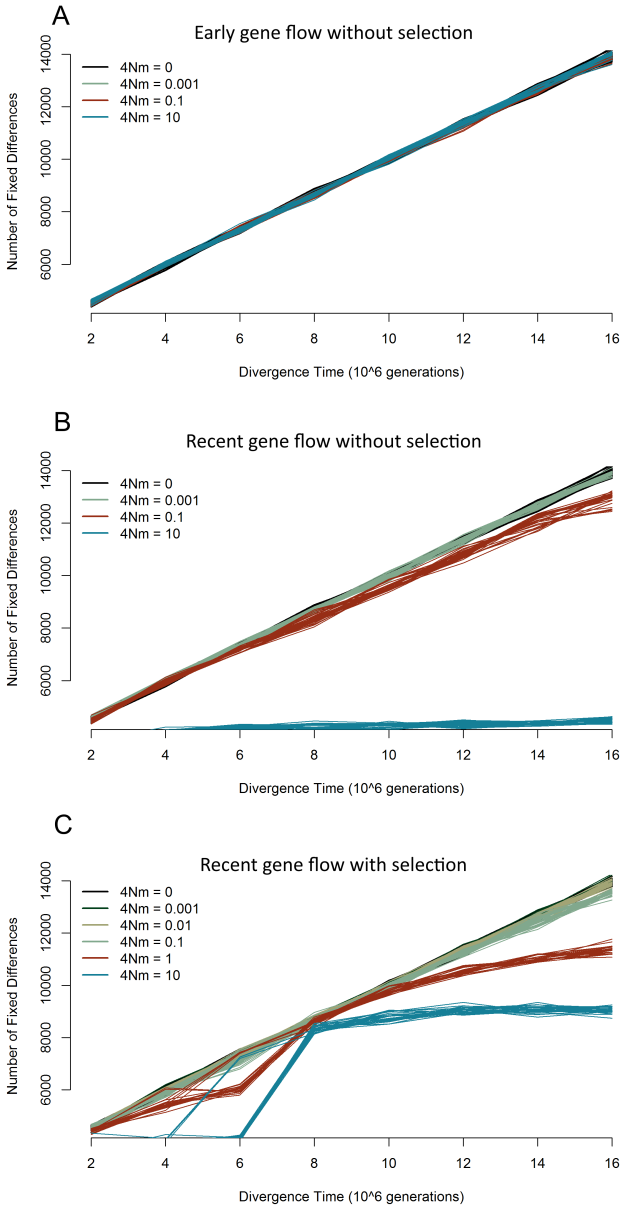

Figure A3: A,B: For simulations without selection, number of fixed differences between species pairs increases linearly with divergence time with early gene flow and, except at higher gene flow rates, with recent gene flow. C: With selection, the number of fixed differences increased linearly with low recent gene flow but nonlinearly with higher gene flow.

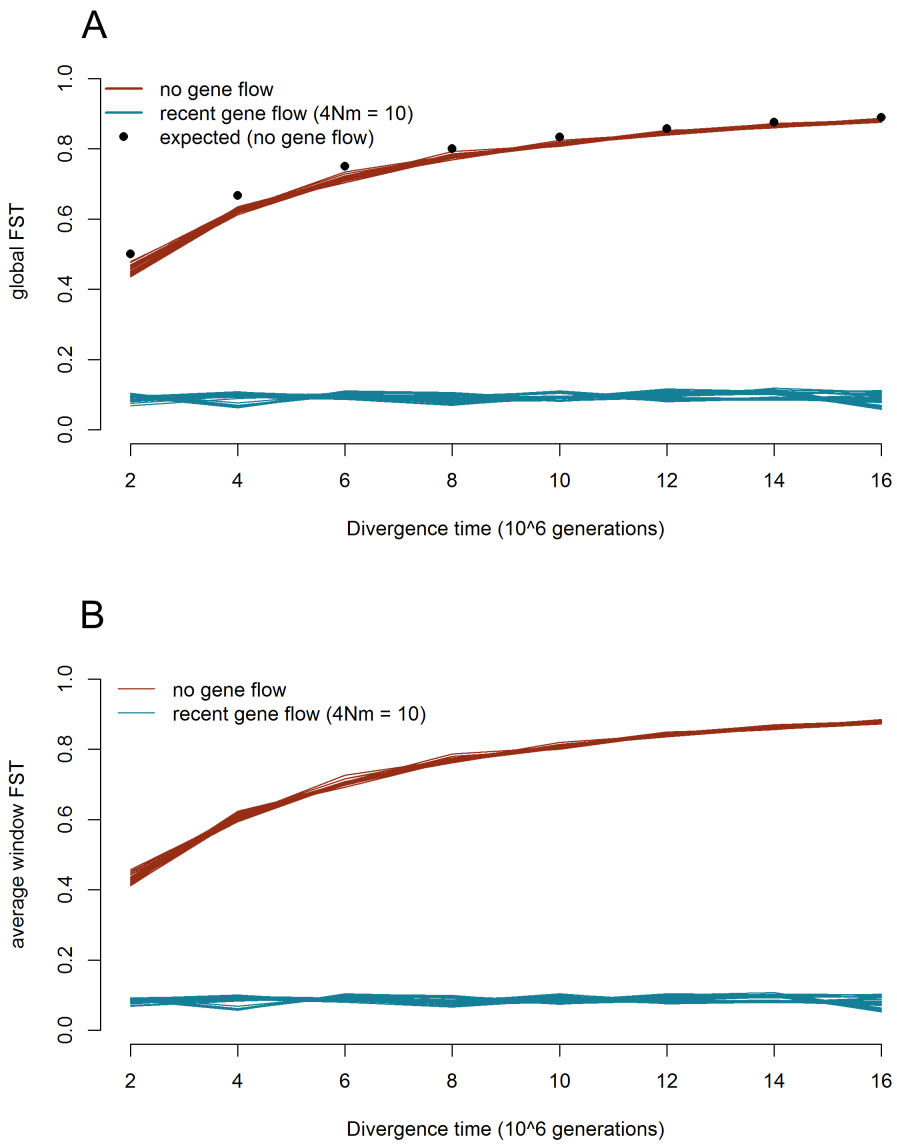

Figure A4: A: Genome-wide F_{ST} increases with divergence time when no gene flow is allowed, but stays constant if high recent gene flow (4Nm = 10) occurs. B: Mean window F_{ST} shows the same pattern.

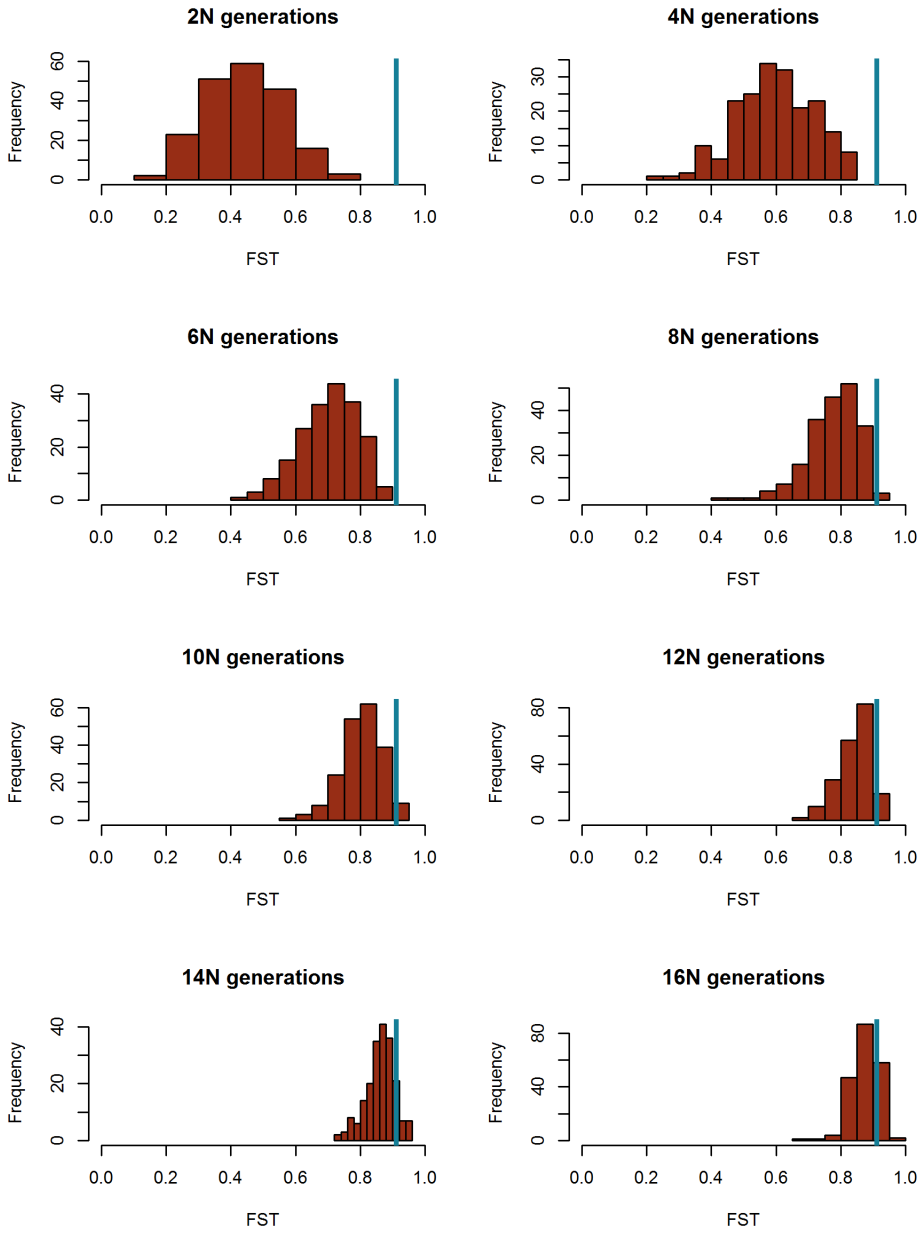

Figure A5: Distribution of F_{ST} values of 500 bp windows in a representative run of the no gene flow, no selection simulation for species pairs of different ages. Blue vertical lines indicate the 95% quantile of all F_{ST} values across all divergence times.

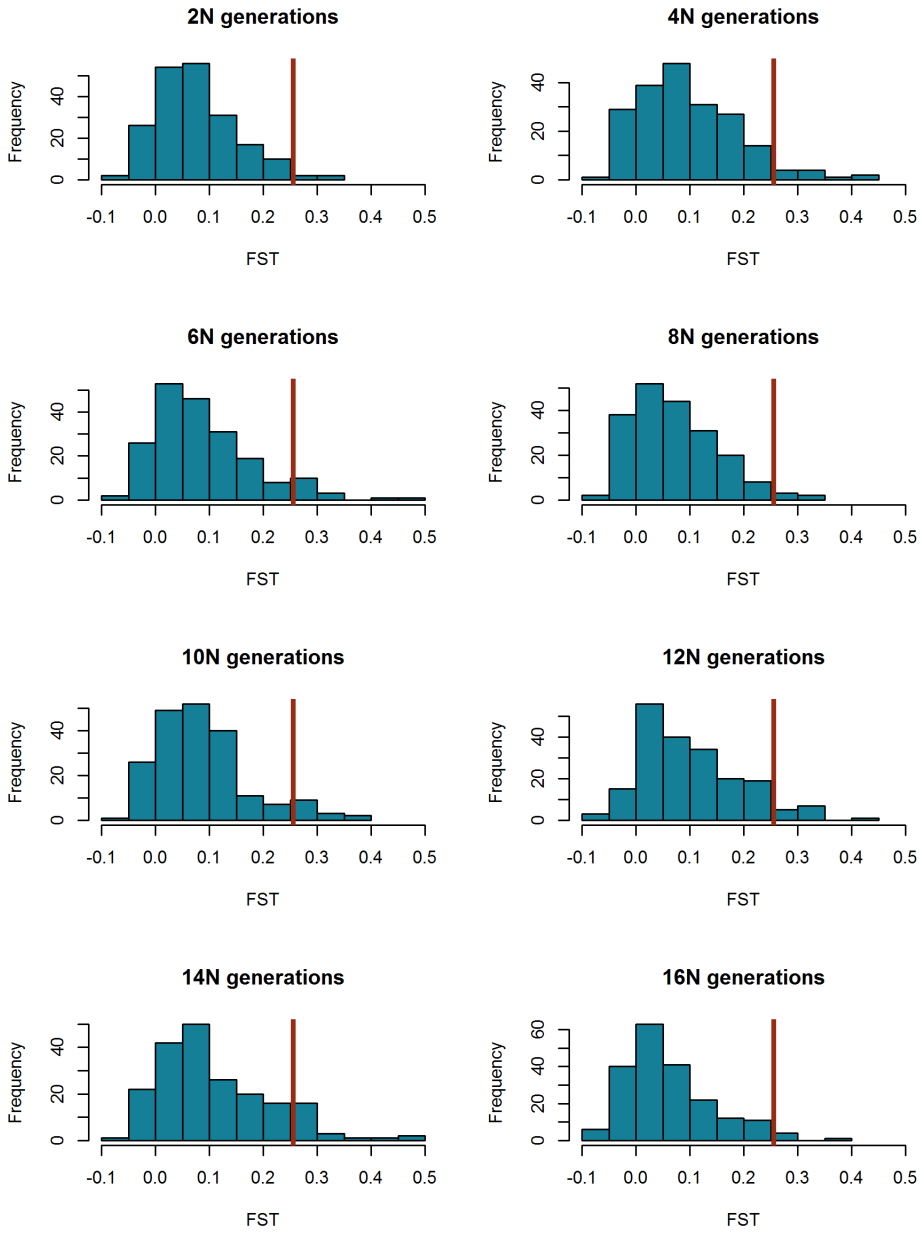

Figure A6: Distribution of F_{ST} values of 500 bp windows in a representative run of the simulations with the highest unidirectional gene flow (4Nm = 10) and no selection for species pairs of different ages. Red vertical lines indicate the 95% quantile of all F_{ST} values across all divergence times.

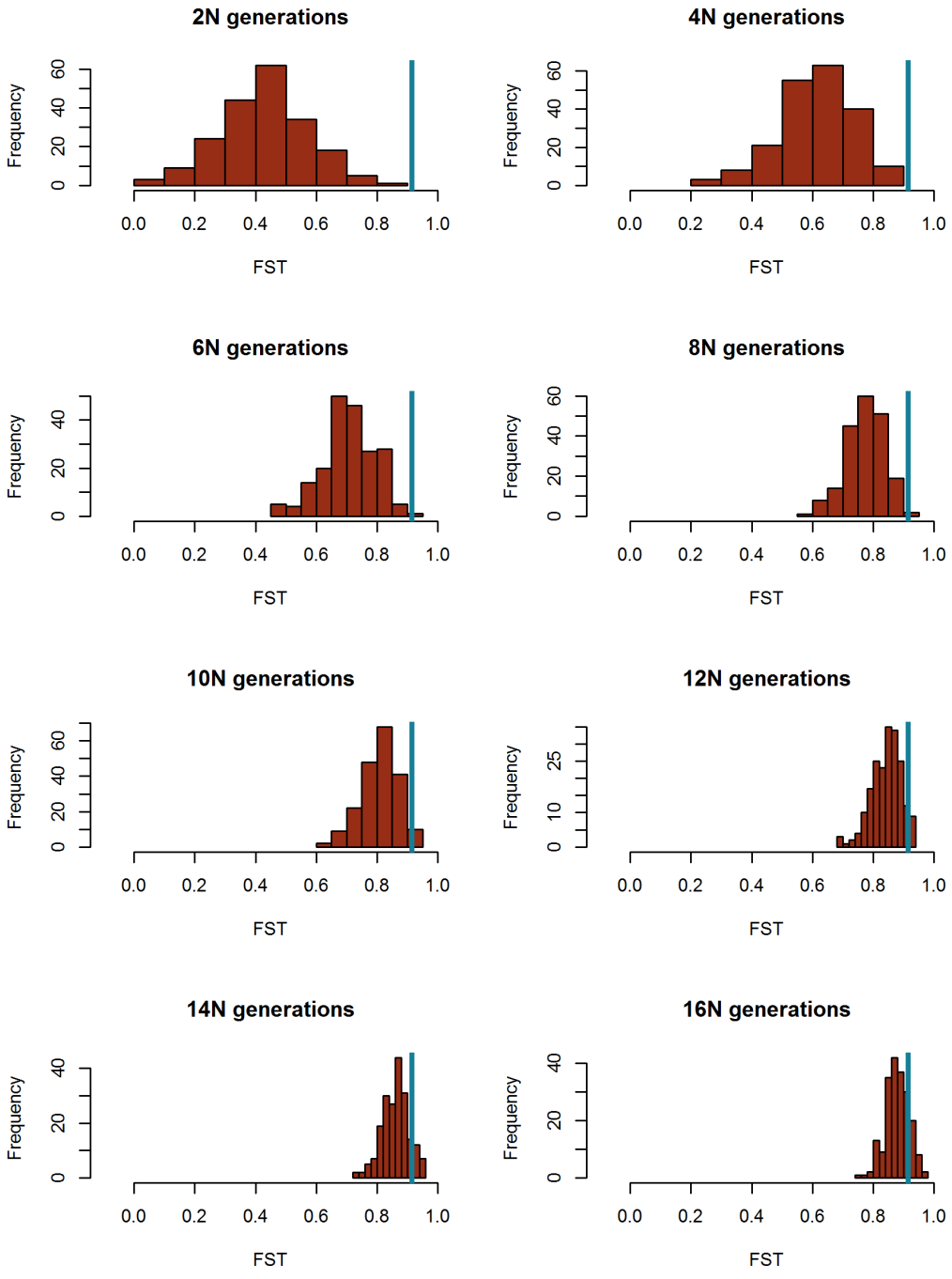

Figure A7: Distribution of F_{ST} values of 500 bp windows in a representative run of the simulations with no gene flow and divergent selection for species pairs of different ages. Blue vertical lines indicate the 95% quantile of all F_{ST} values across all divergence times.

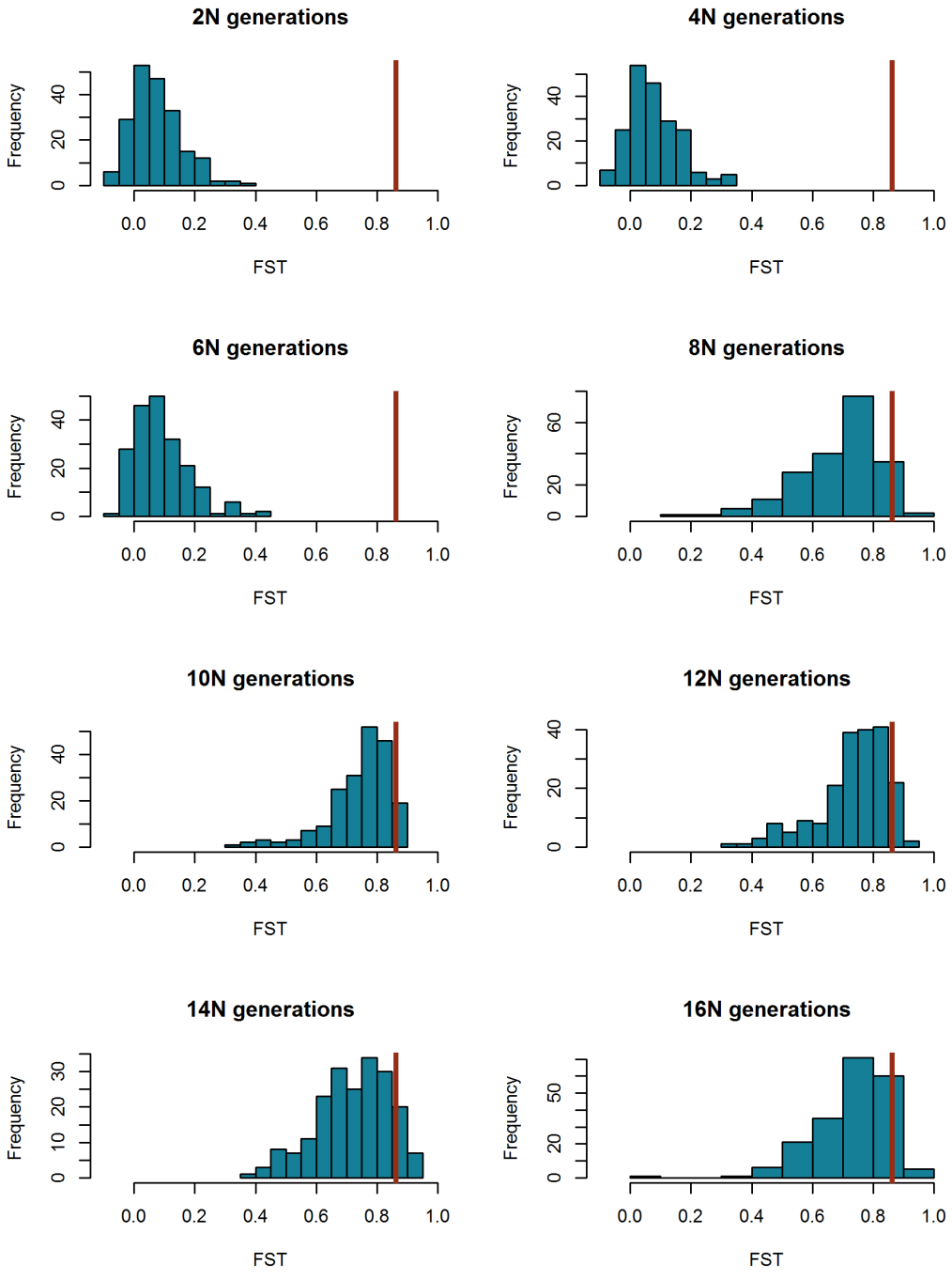

Figure A8: Distribution of F_{ST} values of 500 bp windows in a representative run of the simulations with the highest unidirectional gene flow (4Nm = 10) and divergent selection for species pairs of different ages. Red vertical lines indicate the 95% quantile of all F_{ST} values across all divergence times.

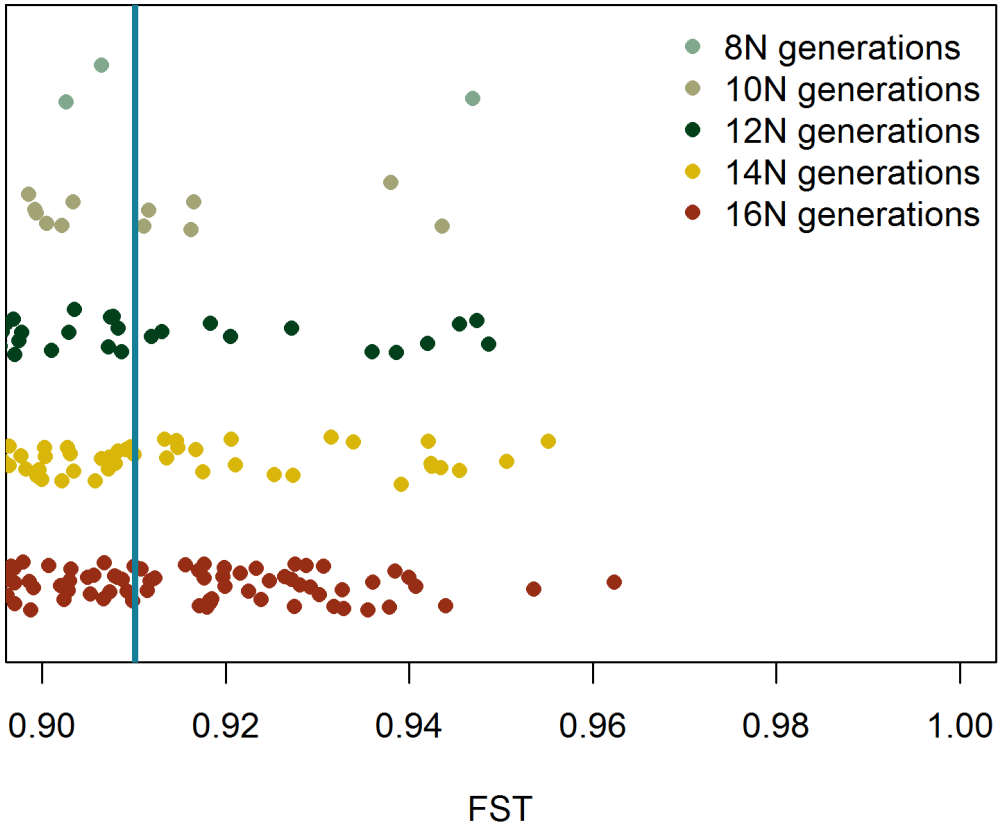

Figure A9: Upper tails of F_{ST} distributions showing number of windows above the 95th percentile threshold in each species pair. Each circle represents one 200bp window. Vertical blue line indicates the 95th percentile (F_{ST} = 0.910). Points to the right of this threshold were deemed outlier windows. Data are from a simulation with no gene flow or selection and N = 10^6. Three younger divergence times (2N, 4N, and 6N generations) are not shown because no windows exceeded the threshold.

APPENDIX B

SUPPLEMENTAL MATERIAL FOR CHAPTER 3

Survival and behaviour of painted butterflies

Females were randomly assigned to two treatments: forewing bands painted as described in the main text or unpainted. We did not compare painting the forewing band vs. outside forewing band in this pilot study, as the intentation was to determine whether the handling and paint itself altered the butterflies' activity levels and survival. Both sets of females, however, had identifying marks written on their wings with an indelible marker, as had all butterflies used in the larger study.

We checked the cages where these butterflies were housed daily and collected any that had died. We recorded whether each female survived for at least one week post-treatment. Butterflies were not individually tracked for longer because the mate choice trials would only be conducted one to two days after painting. Of 14 painted butterflies, 3 died within a week of treatment; of 18 unpainted females, 1 died within a week. These proportions do not differ significantly. We note, also, that in the mate choice experiment, we did not test females that were behaving unusually (e.g. struggling to fly) on the day of the experiment (<5 individuals).

We observed behaviour of 9 painted and 15 unpainted butterflies for 15 minutes the day after they were treated. We recorded what the female was doing at the beginning of each 30 second interval: perching, flapping wings slowly while perched, flying, walking, and feeding. Because the butterflies spent most of their time perched and the other behaviours had only a few observations per individual, we totalled all behaviours except perching and slowly flapping wings as "active" and tested whether the number of active intervals differed between treatments with a Mann-Whitney U-test. There was no significant difference in activity levels between painted and unpainted butterflies (Figure B5, $U = 48.5$, $p = 0.26$).

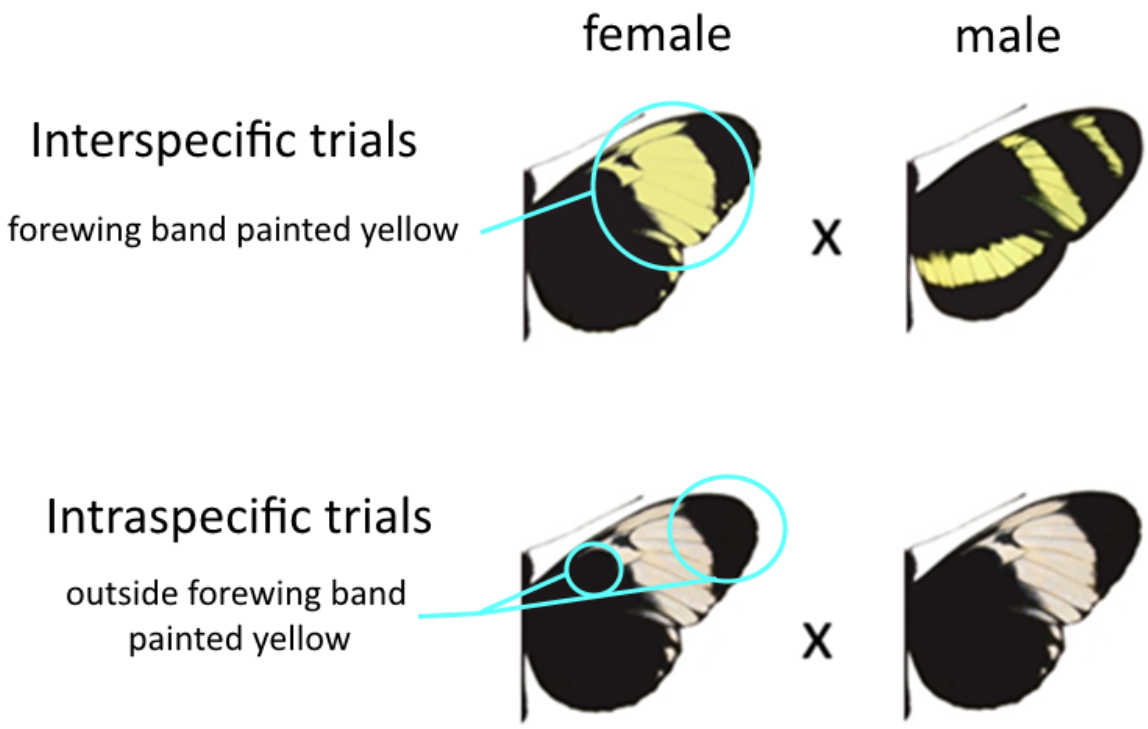

Figure B1: Experimental design: *H. cydno* females paired with *H. pachinus* males had their white forewing band painted yellow with a Copic YG21 Anise paint pen; those paired with *H. cydno* males had the black part of their forewing painted as a control.

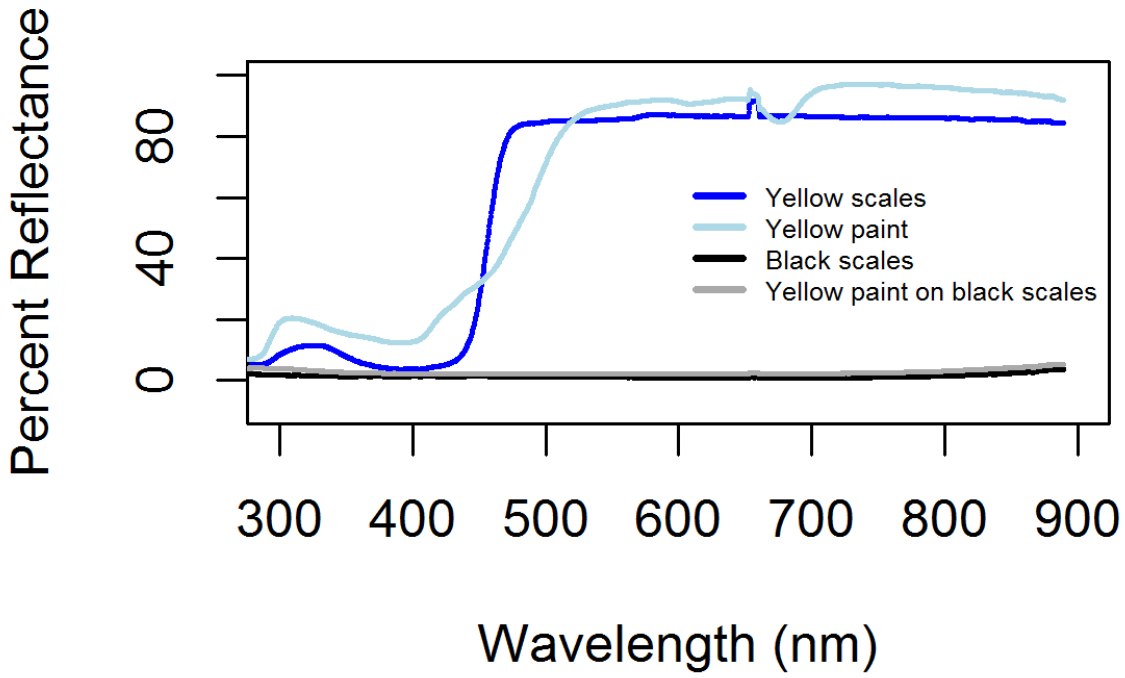

Figure B2: Reflectance spectra of painted and unpainted wing parts. A yellow morph *H. cydno alithea*, which has the same yellow pigment as *H. pachinus* (3-hydroxy-DL-kynurenine), was used as the unpainted yellow sample, and a white morph *H. c. alithea* was painted with a Copic YG21 Anise paint pen as the painted sample.

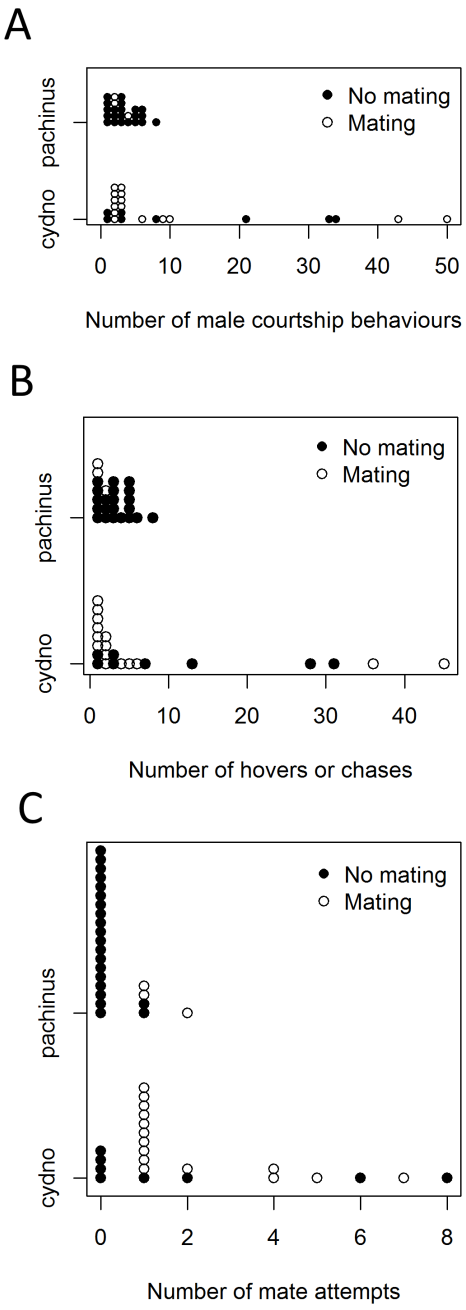

Figure B3: Courtship behaviours of *H. cydno* and *H. pachinus* males. A: Total courtship behaviours. B: Hovers or chases. C: Mate attempts. White dots: trials that ended in mating. Black dots: trials that did not end in mating. See Table 3.1 for descriptions of behaviours. Some sample sizes differ from those in Table 3.2 of the main text because not all behaviours were recorded in a few early trials.

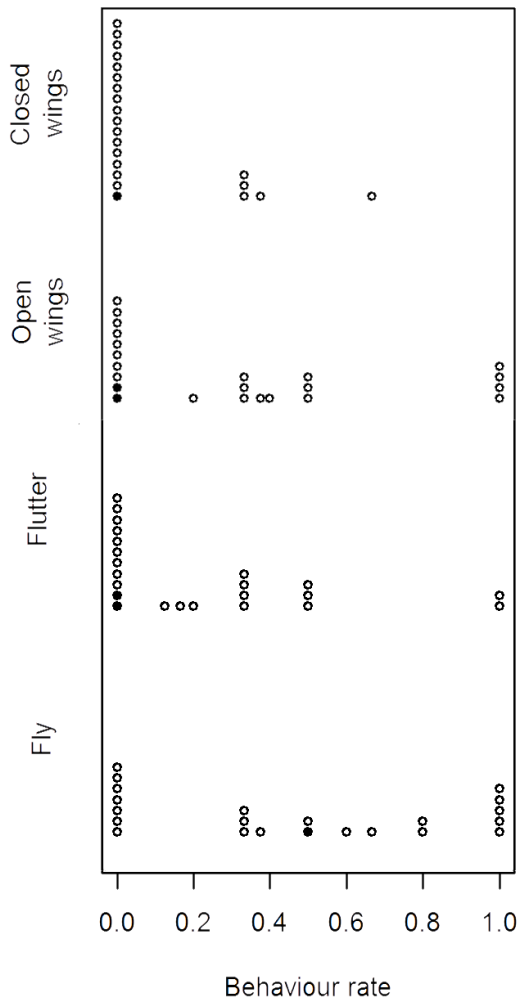

Figure B4: Rates of female behaviours (number of times female performed behaviour divided by number of male courtship behaviours) for interspecific trials (*H. cydno* females and *H. pachinus* males). Black dots: trials that ended in mating. White dots: trials that did not end in mating. Some sample sizes differ from those in Table 3.2 because not all behaviours were recorded in a few early trials.

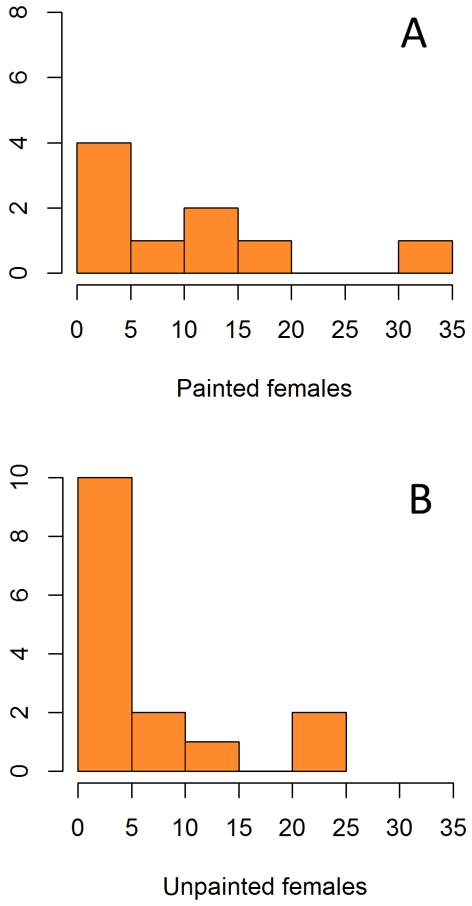

Figure B5: Number of active 30-second intervals of painted (A) and unpainted (B) butterflies.

APPENDIX C

SUPPLEMENTAL MATERIAL FOR CHAPTER 4

Table C1: Studies and species/subspecies pairs used in the meta-analysis.

Study	Species pair
Estrada and Jiggins 2008	*H. melpomene cythera x H. erato cyrbia* *H. melpomene plesseni x H. erato notabilis*
Giraldo et al. 2008	*H. timareta florencia x H. melpomene malleti*
Jiggins et al. 2001	*H. cydno chioneus x H. melpomene melpomene* *H. melpomene rosina x H. cydno chioneus* *H. melpomene rosina x H. melpomene melpomene*
Jiggins et al. 2004	*H. melpomene cythera x H. melpomene malleti* *H. melpomene malleti x H. melpomene melpomene* *H. melpomene malleti x H. melpomene plesseni* *H. melpomene plesseni x H. melpomene melpomene*
Kronforst et al. 2006b	*H. cydno galanthus x H. pachinus* *H. cydno alithea white x yellow*
Kronforst et al. 2007	*H. cydno galanthus x H. pachinus*
Mavárez et al. 2006	*H. cydno cordula x H. melpomene melpomene* *H. heurippa x H. cydno cordula* *H. heurippa x H. melpomene melpomene*
McMillan et al. 1997	*H. erato cyrbia x H. himera*
Mérot et al. 2015	*H. timareta thelxinoe x H. melpomene amaryllis*
Merrill et al. 2011b	*H. melpomene amaryllis x H. melpomene agalaope* *H. melpomene rosina x H. cydno chioneus*
Merrill et al. 2014	*H. erato cyrbia x H. himera*
Munoz et al. 2010	*H. erato chestertonii x H. erato venus*
Naisbit et al. 2001	*H. melpomene rosina x H. cydno chioneus*
Sánchez et al. 2015	*H. cydno cordula x H. timareta florencia* *H. cydno cordula x H. timareta linaresi* *H. melpomene malleti x H. cydno cordula* *H. melpomene malleti x H. timareta florencia* *H. melpomene malleti x H. timareta linaresi* *H. timareta florencia x H. timareta linaresi*
This study	*H. cydno galanthus x H. pachinus*