Title

The role of sexual isolation during rapid ecological divergence: evidence for a new dimension of isolation in *Rhagoletis pomonella*

Running title

Sexual isolation in early divergence

Authors and affiliations

Alycia C. R. Lackey[1,2,5], Alyssa C. Murray[2,3], Nadia A. Mirza[2,4], Thomas H. Q. Powell[2]

[1] University of Louisville, Louisville, KY 40292

[2] Binghamton University, Binghamton, NY 13902

[3] Present Address: Tufts University, Medford, MA 02153

[4] Present Address: Boston University, Boston, MA 02118

[5] corresponding author, alycia.lackey@louisville.edu

Author contributions

ACRL and THQP conceived of the study and experimental design. ACRL, ACM, and NAM conducted the experiment. ACRL and ACM analyzed the data. ACRL wrote the manuscript with contributions from THQP, ACM, and NAM.

Acknowledgements

This work was funded by an NSF grant (NSF 1639005) to THQP and D A. Hahn. Thanks to S. Berlocher and C. Giers for help with access to collection sites in Urbana, Illinois. We greatly appreciate help with lab work and fly husbandry from M. Molina Mera, E. Romeo, D. Fama, I. Pyatetsky, A. Ahmed, A. Dukat, S. Maung, and P. Deneen.

Data accessibility statement

Upon acceptance, data will be archived in Dryad.

The role of sexual isolation during rapid ecological divergence: evidence for a new dimension of isolation in *Rhagoletis pomonella*

Abstract

The pace of divergence and likelihood of complete speciation may depend how and when different types of reproductive barriers evolve. After initial reproductive barriers evolve, questions remain about how subsequently evolving barriers may facilitate additional divergence and potential speciation. We tested for the presence of sexual isolation (reduced mating between populations due to divergent mating preferences and traits) in *Rhagoletis pomonella* flies, a model system for incipient ecological speciation. We measured the strength of sexual isolation between two very recently diverged (~170 years) sympatric populations, adapted to different host fruits. We found that sexual isolation was significantly stronger than expectations of random mating. Thus, sexual isolation may play an important role in reducing gene flow allowed by earlier-acting ecological barriers. We also found that sexual isolation was markedly asymmetric between the sexes of each population. Lastly, we tested how warmer temperatures predicted under climate change could alter sexual isolation and found that mating interactions were sensitive to temperature experienced during development. Our findings provide a window into the early divergence process and the role of sexual isolation after initial ecological divergence, in addition to examining multiple factors that could shape the likelihood of further divergence.

Keywords reproductive isolation, sexual isolation, speciation, mating, asymmetry

Introduction

During the process of ecological speciation, adaptation to different environments can rapidly drive divergence (Schluter 2000, Nosil 2012). Yet, while ecological divergence can quickly differentiate populations, the speciation process frequently remains incomplete (Nosil et al. 2009, Marques et al. 2019), or reversible (Seehausen et al. 1997, Lackey and Boughman 2017, Zhang et al. 2019). How rapidly or completely divergence proceeds depends on the strengths and types of reproductive barriers that evolve and when these barriers evolve during divergence (Coyne and Orr 2004, Lowry et al. 2008, Dopman et al. 2010, Schemske 2010, Lackey and Boughman 2017). Thus, understanding how multifaceted reproductive isolation develops along the speciation continuum following initial ecological divergence has important implications for the tempo of diversification.

In ecological speciation, barriers under direct divergent selection evolve first, and subsequent barriers can evolve independently or as a by-product of divergent adaptation (Schluter 2001, Dieckmann and Doebeli 2004, Rundle and Nosil 2005). Determining how subsequent barriers evolve is important for predicting how rapidly divergence can occur (Smadja and Butlin 2011). Divergence proceeds most rapidly when reproductive isolation occurs as a direct consequence of divergent selection (Servedio et al. 2011). Such divergent selection might result in the evolution of a single strong barrier to gene flow, or multiple barriers might evolve. For instance, local adaptation to different habitats can cause a performance trade-off that limits fitness in alternative environments, which should strengthen divergent habitat use (i.e., habitat isolation; Rice and Hostert 1993, Berlocher and Feder 2002). Divergent adaptation can also result in other barriers, including immigrant inviability, temporal isolation, sexual isolation, or ecological selection against hybrids (Coyne and Orr 2004, Nosil 2012, Servedio and Boughman 2017). Additional reproductive barriers may evolve as a by-product of divergent selection via pleiotropy or hitchhiking, or evolve independently of divergent adaptation (Rice and Hostert 1993, Smadja and Butlin 2011).

Theoretical and empirical work predicts that speciation is most likely to occur when divergent selection acts on both mating and non-mating traits (van Doorn et al. 2009, Maan and Seehausen 2011, Weissing et al. 2011, Wagner et al. 2012). Indeed, sexual isolation, reduced mating between populations due to divergent mating traits and preferences, can play an essential role during the speciation process. Sexual isolation often evolves early in divergence and can strongly facilitate speciation (Coyne and Orr 2004, Mendelson et al. 2007, Lackey and Boughman 2017). Sexual isolation is more likely to facilitate divergence when it coincides with other barriers (Butlin and Smadja 2018). Sexual isolation often occurs in conjunction with ecological isolation, and this combination characterizes many cases of rapid speciation (Boughman 2002, Ritchie 2007, Seehausen et al. 2008, Maan and Seehausen 2011). Ecological and sexual isolation may evolve rapidly in concert when direct selection acts on ecological and sexual traits (e.g., habitat choice and environmentally-dependent signal production or fitness; McNett and Cocroft 2008, Boughman and Svanback 2017, Maan and Seehausen 2011, Nosil 2012, Safran et al. 2013, Scordato et al. 2014, Servedio and Boughman 2017). Additionally, the same trait(s) may shape both ecological and sexual barriers (Jiggins et al. 2001, Servedio et al. 2011). When sexual isolation occurs along with ecological isolation, it provides an opportunity to understand the relative roles and interdependence of these barriers, reveal the mechanisms currently shaping population differentiation, and potentially understand the origin and evolution of reproductive isolation. This is particularly true when studying populations in early stages of divergence and comparing them to populations at later stages along the speciation continuum.

Predicting how quickly or completely isolation can evolve also involves evaluating how potential asymmetries in the strength of isolation between populations shape gene flow. Asymmetric reproductive isolation can result from differences between populations in the strength of selection on parental phenotypes or differences in fitness costs for hybrids that are stronger in one direction (Kaneshiro 1980, Arnold et al. 1996, Tiffin et al. 2001, Turelli and Moyle 2007, Kuwajima et al. 2010, Ribardiere et al. 2019). Strong asymmetries may limit or reverse divergence (Arnold et al. 1996, Servedio and Kirkpatrick 1997, Chunco et al. 2007). While asymmetries may be common early in divergence, the extent of asymmetries may diminish as divergence proceeds and selection acts more symmetrically on each population or as incompatibilities arise (Turelli and Moyle 2007, Lackey and Boughman 2017). Even if asymmetries persist at later stages of divergence, their effects can be offset by complementary asymmetries in another barrier (Wade et al. 1995, Kitano et al. 2007, Takami et al. 2007).

While divergent ecological selection can rapidly generate reproductive isolation, environmental sensitivity of reproductive barriers has important consequences for gene flow and the potential for distinct species to evolve and persist. Reproductive isolation that evolves due to divergent ecological selection may weaken if environmental differences decrease (Seehausen et al. 1997, Grant and Grant 2008, Heath et al. 2010, Vonlanthen et al. 2012, Lackey and Boughman 2017). Sexual isolation may be particularly sensitive to environmental changes when differences in mating preferences and traits evolved due to environmental differences (Seehausen et al. 1997, Fisher et al. 2006, Ward and Blum 2012, Lackey and Boughman 2013).

Here, we leveraged a well-established study system in ecological speciation, *Rhagoletis* flies, to evaluate how multifaceted reproductive isolation may evolve, particularly early in divergence. Populations of *Rhagoletis* flies have diverged to adapt to a wide variety of fruiting host plants (Berlocher 2000). One pair of very recently diverged (~170 generations) populations of *Rhagoletis pomonella* have differentially adapted to apple and hawthorn host plants (Walsh 1861, Bush 1966, Linn et al. 2003, Feder et al. 2010, Powell et al. 2020). While habitat and temporal isolation strongly limit gene flow and maintain consistent allele frequency differences between sympatric populations (Feder et al. 1988, Michel et al. 2010, Powell et al. 2013), reproductive isolation remains incomplete. Flies from different host-associated populations can still encounter each other, and mark recapture estimates for apple and hawthorn flies indicate gross migration of ~6% in sympatry (Feder et al. 1994). In this system, much less is known about the strength and evolutionary underpinnings of reproductive barriers that may not be under direct divergent ecological selection. Questions remain as to the presence and strength of sexual isolation in *R. pomonella* as well as the potential forces that might underlie this barrier. Across the genus of *Rhagoletis*, previous work suggests that sexual isolation is strong between highly divergent species pairs but absent or weak between closely related taxa (Hood et al. 2012).

Given how commonly sexual isolation plays an important role both early in divergence and in the likelihood of speciation, we made a novel extension of this study system to assess the contribution sexual isolation to limiting gene flow. First, we measured sexual isolation between recently diverged, sympatric populations of apple and hawthorn *R. pomonella* flies. Second, we examined potential asymmetries in sexual isolation by measuring the contribution of each sex from each population to overall sexual isolation. Third, we tested whether rearing fly pupae under control and warmed temperature regimes that mimic climate change predictions in the next 50-100 years affected mating interactions with consequences for the strength of sexual isolation or patterns of mating success (i.e., frequency or duration of mating interactions).

Methods

Insect collection and rearing

We collected fruit infested with *Rhagoletis pomonella* flies from apple (*Malus pumila*) and hawthorn (*Crataegus mollis*) trees at a sympatric site in Urbana, Illinois in 2017. We collected apples in mid-August and hawthorns in mid-September. We transported fruit to Binghamton University and maintained fruit at approximately 26°C with 14:10 L:D. We collected larvae that emerged from fruit daily for three weeks, following the natural emergence cycle. Each day, we randomly assigned larvae to two temperature regimes, Control and Warming, described below. We placed larvae into petri dishes with moist vermiculite in environmental chambers (Percival I41VLC9) with their assigned temperature regime for 10 days during the transition into the pupal phase. We then transferred viable pupae into individual 0.2 µl tubes and returned them to their assigned temperature regime until adult flies eclosed in the spring and summer of 2018.

We created temperature regime programs using weekly average minimum, midpoint, and maximum temperatures calculated from soil temperature data from NOAA's National Climatic Data Center (NCDC) from 2007 to 2016 (Watseka, Illinois station: 40.79, -87.76). We used soil temperatures at a depth of 10cm, which is the approximate depth of pupal *R. pomonella* during diapause (Feder 1995). Temperature programs and light:dark cycles replicated natural daily oscillations and weekly changes throughout the year (see Supplemental methods text for detail). We based the Control temperature regime on the 10-year weekly averages. Warming temperature regime set points were all 3°C higher than Control, which falls within the range of expected temperature increases for the Midwest in the next 50-100 years for multiple emission scenarios (Pryor et al. 2013). We monitored pupae daily for eclosion after winter programs.

We housed newly eclosed flies individually in 50 mL Falcon tubes with food (3:1 sugar to yeast hydrolysate mixture, Neilson and McAllan 1964) and water for one day to allow for sclerotization of adult cuticles and wings. Then, flies were assigned to mating trials and painted with randomly assigned marking codes unique to each of 20 individuals within a trial. We used Testors^{TM}(Vernon Hills, Illinois, USA) enamel paint for marking, and we briefly anesthetized flies on carbon dioxide blocks to apply paint. Flies were then housed in clear plastic containers with mesh tops (approximately 1L) in same-sex groups of up to five with food and water *ad libitum* and kept at approximately 26°C and 14:10 L:D cycle.

Mating trials

We used multiple choice mating trails with 5 males and 5 females of each population to test whether copulation is more likely to occur within versus between populations. This design mimics natural conditions where flies aggregate on host plants to mate (Prokopy 1976, Aluja et al. 2001). Trials with multiple males and females allow both sexes to engage in mate choice. Thus, we used this design to measures overall sexual isolation and the contributions of each sex from each population.

We conducted a mating trial once all flies assigned to a trial had reached reproductive maturity (at least 10 days old; Neilson and McAllan 1965). For each trial we assigned 5 males and 5 females of each population (Apple and Hawthorn) reared under the same temperature regime (Control or Warming). While we initially assigned 5 flies of each sex from each population to trials, some trials had 4-6 flies of each sex and population due to early mortality and one case of misassignment. In our analysis, we accounted for sample size variation in expectations of random mating. We conducted 3-hour mating trials in tent-shaped enclosures with clear plastic and white mesh sides (BugDorm2^{TM}, MegaView Science Education Services LTD, Taiwan; 61 x 61 x 61cm). Each tent contained two water and two food stations as well as an apple as a mating stimulus. Both Apple and Hawthorn flies mate readily on and oviposit into apples in lab trials (Linn et al. 2004, Lyons-Sobaski and Berlocher 2009). We introduced flies to the mating arena by allowing them to fly out of their opened housing enclosures. We introduced females first and allowed them to acclimate for 10 minutes before introducing males. We observed up to 4 mating trials concurrently during each 3-hour observation using scan sampling. For every attempted copulation (one fly mounts the other), we recorded copulation duration and identity of the interacting flies using paint marks. Males typically initiate mating by jumping on the female's back (Smith and Prokopy 1982). Females can resist and dislodge males or accept a mating attempt by extending her ovipositor. Because of the time it takes for sufficient insemination to occur, copulations longer than 5 minutes were categorized as successful (Hood et al. 2012). Copulations typically last at least 20 minutes (Smith and Prokopy 1982, Schwarz and McPherson 2007).

Statistical analysis

<u>Sexual isolation</u>

We calculated sexual isolation using the following equation (Sobel and Chen 2014):

$$SI = 1 - 2\left(\frac{H}{C+H}\right) \quad (1)$$

where *H* is the frequency of heterospecific, or between-population, events and *C* is the frequency of conspecific, or within-population events. *SI* ranges linearly from -1 (mating only between populations) to 0 (random mating) to 1 (mating only within populations). To account for variation in the number of males and females of each population in each trial, we calculated expected copulations for each pair type (Apple female x Apple male, Apple female x Hawthorn male, Hawthorn female x Apple male, Hawthorn female x Hawthorn male; abbreviated AA, AH, HA, HH) based on random mating null expectations. For each sex of each population, we divided the total number of copulations that group had with flies of the opposite sex from either population with 50:50 mating expectations given the number of Apple males and Hawthorn males in a trial. For example, if Apple females in a trial had 4 copulations, and there were equal numbers of Apple (5) and Hawthorn (5) males, then the expected number of copulations given random mating would be 2 Apple female x Apple male and 2 Apple female x Hawthorn male. If there were unequal numbers of males (5 Apple, 4 Hawthorn), then the expected number of copulations would be 2.22 Apple female x Apple male and 1.78 Apple female x Hawthorn male. We used these expected copulations in the following equation (Sobel and Chen 2014):

$$SI = 1 - 2\left(\frac{\frac{H_{obs}}{H_{exp}}}{\frac{C_{obs}}{C_{exp}} + \frac{H_{obs}}{H_{exp}}}\right), \quad (2)$$

where observed events (*obs*) were divided by expected events (*exp*). We calculated 95% confidence intervals for total sexual isolation using 127 total copulations as the sample size. To calculate 95% confidence intervals for the contributions of each sex to sexual isolation, we used the following sample sizes: 53 copulations with Apple females, 70 copulations with Apple males, 74 copulations with Hawthorn females, and 57 copulations with Hawthorn males.

We used a linear mixed model to complement the sexual isolation calculations. This model tested the fixed effect of pair type (AA, AH, HA, HH) and the random effect of trial on the proportion of observed out of expected copulations. We performed this analysis and the following analyses in R 4.0.5 (R Core Team 2020). We used packages lme4 (Bates et al. 2015) and emmeans (Lenth 2021) to test the model and calculate the least-squared means and contrasts. When main effects or interactions were significant, we ran post-hoc tests of pairwise differences using least squares means and a false discovery rate (FDR) p-value adjustment (Benjamini and Hochberg 1995, Verhoeven et al. 2005).

Comparing prezygotic isolating barriers

To place the strength of sexual isolation in context to other prezygotic barriers linked to divergent adaptation to different host plants, we measured the strength of temporal and habitat isolation from existing data. Data for temporal isolation were calculated for Apple and Hawthorn flies reared under control temperatures (Lackey et al. *in prep*). For habitat isolation, we used data from fruit volatile preferences in flight tunnels (Linn et al. 2003). After emergence, flies may travel several kilometers to locate host plants, and fruit volatiles are the major long-range stimulus attracting flies (Maxwell and Parsons 1968, Linn et al. 2003). We calculated 95% confidence intervals for each barrier. Next, we calculated the sequential strength of each barrier ordered by their occurrence in the life cycle (i.e., temporal, habitat, sexual). The sequential strength of each barrier (SS_n) is calculated from its individual strength (RI_n) and the amount of gene flow allowed by earlier-acting barriers (Ramsey et al. 2003, Dopman et al. 2010, Sobel and Chen 2014):

$$SS_n = RI_n \left(1 - \sum_{i=1}^{n-1} SS_i\right). \quad (3)$$

Copulation frequencies

We next tested whether a different metric of mating success, copulation frequencies, differed within and between populations. For sexual isolation, we used ratios of observed copulation frequencies out of expected frequencies based on random mating. Here, we analyzed observed copulation frequencies to test for (a) an overall effect of the occurrence of ‘conspecific’ versus ‘heterospecific’ matings not controlled by expected frequencies, (b) an effect rearing temperature on both copulation frequency overall as well as (c) the interactive effect of rearing temperature on ‘conspecific’ versus ‘heterospecific’ matings. We used a generalized linear model with the number of copulations as the response variable, which fit a Poisson distribution. We tested the fixed effects of within- or between-population mating and rearing temperature, and we tested the random effect of trial.

Copulation duration

We next tested effects of within- versus between-population pair types (AA, AH, HA, HH) and rearing temperature on successful copulation duration, which affects both sperm transfer and availability for subsequent matings. We square root transformed duration to improve normality for linear modeling. We tested the fixed effects of within- versus between-population pair type and rearing temperature with a random effect of trial.

Results

Sexual isolation

Sexual isolation between Apple and Hawthorn flies was significantly greater than expectations of random mating, where isolation is zero (SI = 0.15 [95%CI: 0.21 - 0.09], Figure 1). From the perspective of each sex of each population, Apple females and Hawthorn males had stronger sexual isolation (SI = 0.23 [0.34, 0.11] and 0.26 [0.38, 0.15], respectively) than Apple males and Hawthorn females (SI = 0.06 [0.12, 0.006] and 0.04 [0.09, -0.004], respectively, Figure 1).

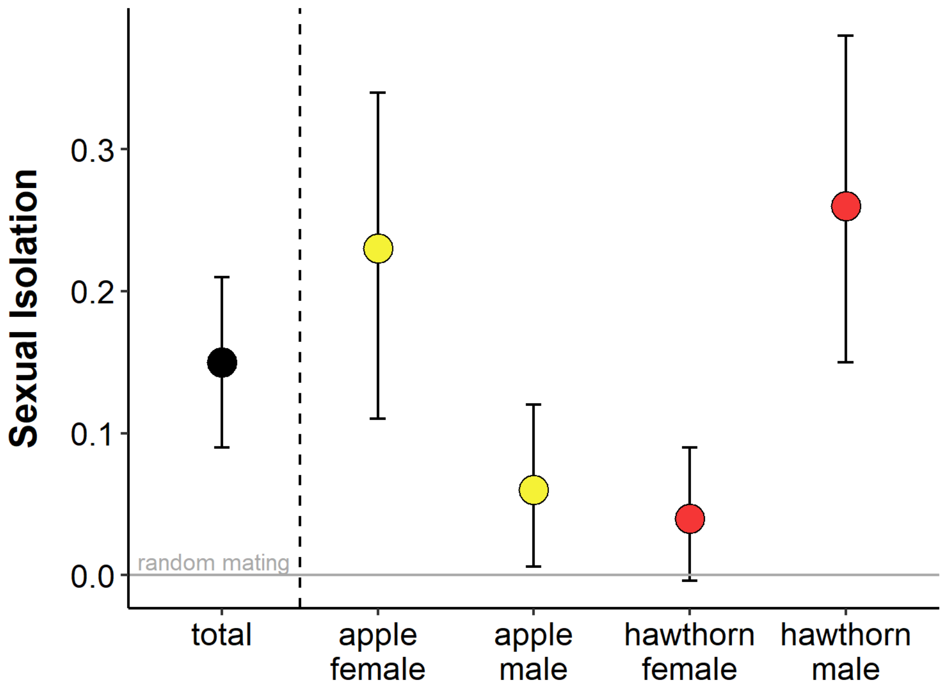

Figure 1. Total sexual isolation and contributions of each sex from each population. The dashed vertical line separates total sexual isolation from contributions of each sex from each population. Circles are point values with 95% CIs. The horizontal grey line at 0 indicates random mating, and positive values indicate greater mating within populations than between.

The results from our linear mixed model support our calculations of sexual isolation. The proportion of observed out of expected copulations was greater for within-population pairs than between-population pairs for Apple females and Hawthorn males (Af-Am > Af-Hm: estimate = 0.45, s.e. = 0.20, t = 2.23, p = 0.0276; Hm-Hf > Hm-Af: estimate = 0.53, s.e. = 0.23, t = 2.35, p = 0.0200; though both effects were marginal after correction with false discovery rate, both p_{FDR} = 0.0552). In contrast, the proportion of observed out of expected copulations did not differ between within- and between-population pairs for Apple males and Hawthorn females (Am-Af = Am-Hf: estimate = 0.13, s.e. = 0.21, t = 0.61, p = 0.54, p_{FDR} =

0.69; Hf-Hm = Hf-Am: estimate = 0.08, s.e. = 0.21, t = 0.40, p = 0.69, p_{FDR} = 0.69). The random effect of trial was negligible with both a variance and standard deviation of approximately 0.

Copulation frequencies

Copulation frequencies did not differ between within- and between-population pairs. For populations with strong and symmetric sexual isolation, we would expect greater frequencies of within-population than between-population matings. However, given the asymmetric strength of isolation between the sexes, it makes sense that we did not a difference in the frequency of within- versus between-population matings.

Rearing temperature significantly affected copulation frequencies (model estimate = 0.76, s.e. = 0.26, z = 2.93, p = 0.0034). Flies reared under Control temperature regimes copulated more frequently (mean = 3.43, 95% CI: 2.72 – 4.35) than flies reared under Warming temperature regimes (mean = 1.64, 95% CI: 1.23 – 2.19). Again, the random effect of trial was negligible.

Copulation duration

Copulation duration was affected by an interaction between rearing temperature and pair type (model effect = 13.40, s.e. = 5.49, df = 115, t = 2.44, p = 0.0162; Figure 2). Between-population copulations in Warming (lsmean = 3047 s, 95% CI: 2313 – 3881) were longer than both within-population copulations in Warming (lsmean = 1953 s, 95% CI: 1414 – 2591; contrast: t = 2.66, p = 0.0089, p_{FDR} = 0.0178) and between-population copulations in Control (lsmean = 1672 s, 95% CI: 1149 – 2304; contrast: t = 4.94, p = 0.0062, p_{FDR} = 0.0178).

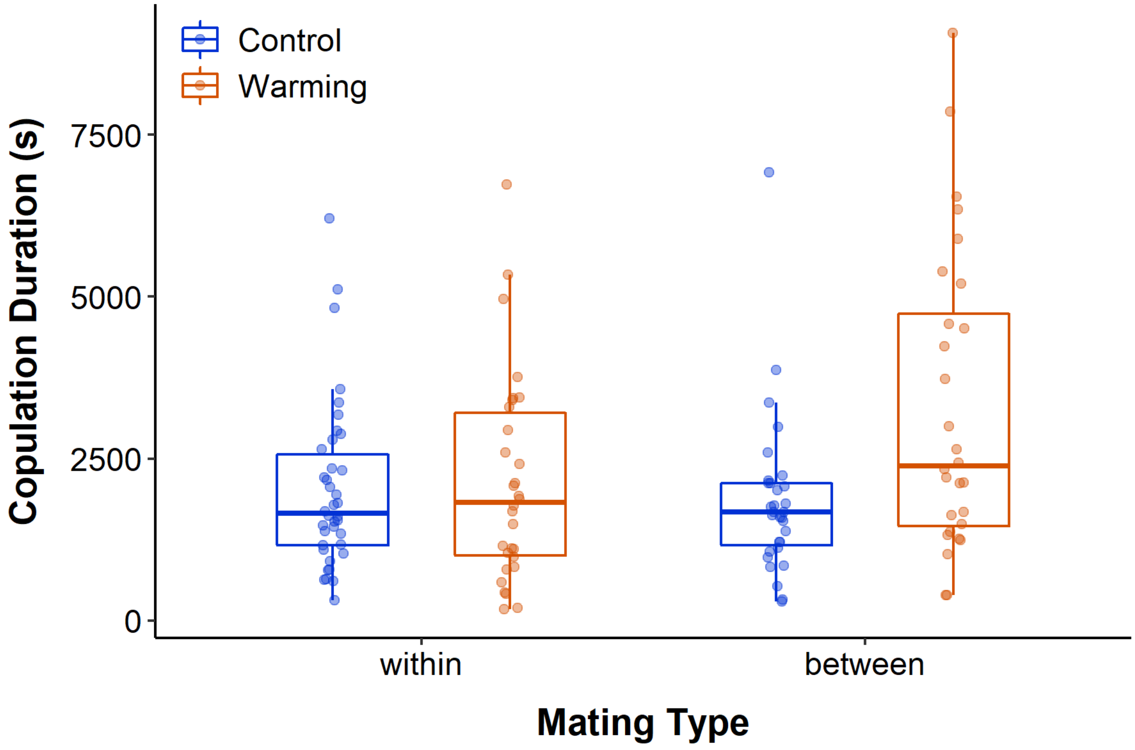

Figure 2. Copulation duration (in seconds) for within- and between-population matings for flies reared in Control or Warming temperature regimes. Dots show durations of each copulation layered over boxplots.

Comparing prezygotic isolating barriers

Individual strengths of isolating barriers estimate the proportion of gene flow limited by each barrier if acting alone. Temporal isolation was moderate in strength (RI = 0.44, 95% CI: 0.31 – 0.56). Habitat isolation, as measured by attraction preference to host fruit volatiles, was the strongest of the three barriers we estimated (RI = 0.87, 95% CI: 0.83 – 0.92). Sexual isolation was relatively weaker than the other barriers (RI = 0.15, 95% CI: 0.08 – 0.21), though significantly stronger than expectations of random mating (RI = 0). The sequential strengths of isolating barriers ordered as each barrier occurs in the life cycle and estimate the proportion of gene flow limited by that barrier given the gene flow allowed by earlier-acting barriers. Together, temporal and habitat isolation were estimated to limit 93% of potential gene flow. Of the remaining 7% of gene flow, sexual isolation would further reduce gene flow by 1%.

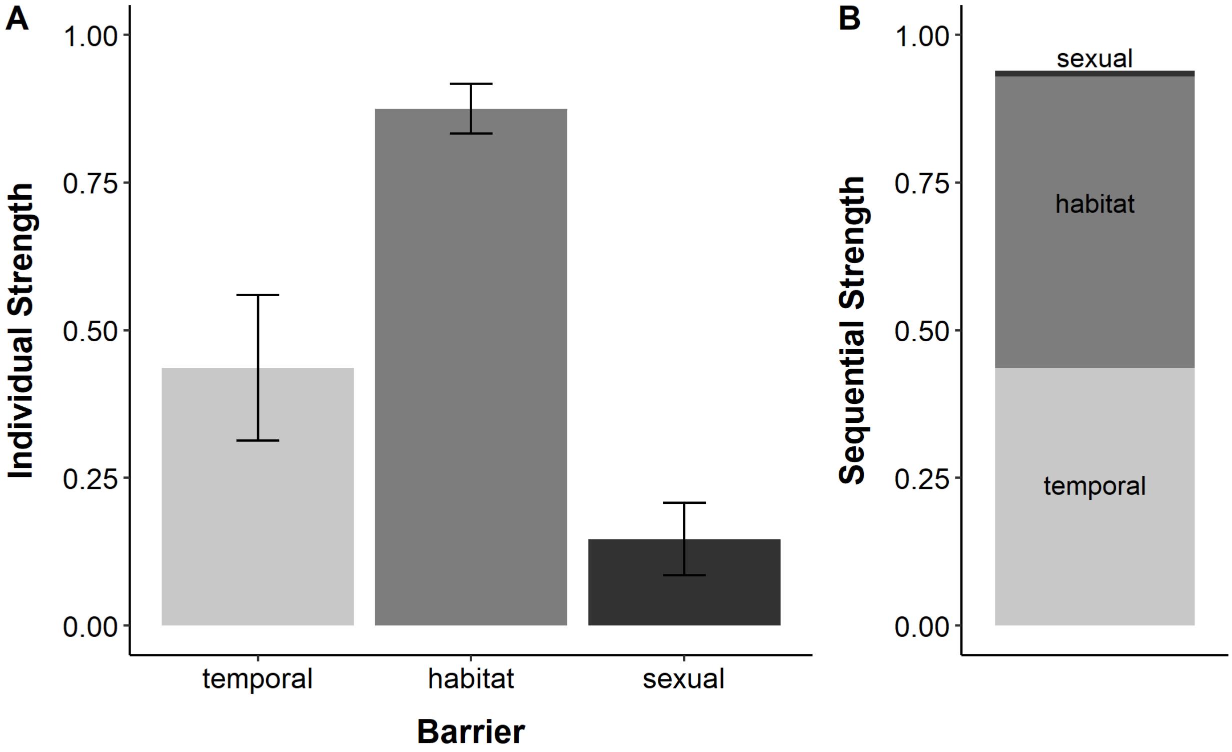

Figure 3. (A) Individual and (B) sequential strengths of three prezygotic barriers. Error bars in A are 95% confidence intervals. Values for barrier strengths and 95% CIs are provided in Supplemental Table 1.

Discussion

A single source of divergent selection can drive rapid and extensive levels of divergence between populations, but this alone is often insufficient to complete speciation (Nosil et al. 2009, Thibert-Plante and Hendry 2011, Kautt et al. 2020). Studying populations early in the process of divergence provides opportunities to measure reproductive barriers as they accumulate and detect the evolutionary forces producing isolation (Nosil et al. 2005, Merrill et al. 2011, Powell et al. 2014, Hood et al. 2020). In addition to measuring the overall strength of reproductive barriers, determining the strength of asymmetries provides insights into the underlying evolutionary processes and understand the nature of how reproductive isolation evolves (Arnold et al. 1996, Servedio and Kirkpatrick 1997, Lackey and Boughman 2017). Even in later stages of divergence, population differences can degrade and allow extensive gene flow, commonly due to environmental change (Seehausen et al. 1997, Vonlanthen et al. 2012, Lackey and Boughman 2013). Thus, estimating environmental sensitivity of reproductive barriers enables predictions of the stability of divergence in the face of environmental change, which is especially important when divergence is primarily driven by environmental differences.

Here, we tested for the presence of sexual isolation, a barrier often important in early stages of divergence, using a well-established case study of rapid divergence with gene flow. Between two very recently diverged populations of apple and hawthorn flies, we have identified the presence of a new dimension of reproductive isolation that has evolved within ~170 generations. We found (1) that the strength of sexual isolation was significantly greater than expectations of random mating, (2) sexual isolation was asymmetric between the sexes of each population, and (3) that mating interactions were sensitive to temperature experienced during development.

Between apple and hawthorn flies, we provide evidence that sexual isolation could limit approximately 15% of gene flow. While sexual isolation is relatively weaker than habitat and temporal isolation, it may play an important role in restricting the homogenizing effects of gene flow and, thus, facilitate divergence. Considering the sequential and combined effects of multiple barriers, temporal and habitat isolation allow 7% gene flow. Adding sexual isolation reduces potential gene flow to 6%, which is consistent with estimated gross migration in the field, based on mark-recapture studies (6%, Feder et al. 1994). From the perspective of remaining potential gene flow, the 1% increase in total reproductive isolation may represent a biologically meaningful reduction. Our observed effect of sexual isolation cuts the potential remaining gross migration rate by 14% (m = 0.07 to 0.06). Such incremental reductions in migration rates may have considerable consequences for migration-selection equilibria (Yeaman and Whitlock 2011) and may nudge systems closer to “tipping points” after which the pace of divergence increases rapidly to form reproductively isolated species (Flaxman et al. 2014, Nosil et al. 2017, Schilling et al. 2018). Moreover, selection on traits that yield sexual isolation may also increase the extent of genome-wide differentiation, strengthening the likelihood of complete and stable speciation (Nosil and Feder 2012, Kautt et al. 2020).

The current strength of sexual isolation between apple and hawthorn flies suggests an increase in isolation compared to an estimate from 30 years ago that found no sexual isolation between different host-associated populations of *Rhagoletis pomonella* (Smith 1988). Moreover, across *Rhagoletis* species, sexual isolation increases in strength from weak to strong as divergence between species increases (Smith 1988, Schwarz and McPherson 2007, Hood et al. 2012). It is currently unknown whether sexual isolation evolves in association with host adaptation or independently.

In the *R. pomonella* species complex, divergent specialization to different host plants has primarily driven divergence and resulted in ecological reproductive isolation between populations through divergent life history timing and olfactory behavioral responses to fruit volatiles (Berlocher 2000, Linn et al. 2005, Dambroski and Feder 2007, Linn et al. 2012, Mattsson et al. 2021). Moreover, geographic isolation alone may be insufficient for the evolution of sexual isolation; sexual isolation was absent between a pair of populations using the same host plant despite 1.5 million years of geographic isolation (Rull et al. 2010). More generally, adaptation to different environments can result in rapid mating trait divergence via direct selection or as a by-product (Lande and Kirkpatrick 1988, Maan and Seehausen 2011, Nosil 2012, Safran et al. 2013, Boughman and Svanback 2017, Servedio and Boughman 2017).

Of particular importance for understanding how distinct species evolve and persist is to determine how reproductive isolation evolves when it is not the result of direct divergent selection. Which evolutionary processes (i.e., indirect selection, hitchhiking, reinforcement, mutation-order) most commonly underlie the accumulation of these additional reproductive barriers? Sexual isolation can evolve via reinforcement when selection against costly matings between populations favors the evolution of prezygotic isolation (Servedio and Noor 2003). In *R. pomonella*, F_1 hybrids may suffer an ecological fitness disadvantage due to reduced responses to host fruit volatiles critical for locating host fruit for reproduction (Linn et al. 2004). Such fitness costs could favor selection for strong mating discrimination via reinforcement. Sexual isolation could also evolve due to population differences in selection along axes independent of primary ecological differences (e.g., non-ecologically mediated sexual selection or sexual conflict, Turbek et al. 2021, Rundle and Rowe 2018) or via non-selective evolutionary processes (e.g., mutation order, Mendelson et al. 2014). Indeed, species maintenance is more likely when at least some reproductive barriers evolve independently of environmental differences (Coyne and Orr 2004, Lackey and Boughman 2017). In *Rhagoletis*, future work is needed to determine the extent to which sexual isolation may result from ecological or non-ecological factors.

In our study, sexual isolation was asymmetric between the sexes of each population, with one sex mating randomly and the other sex mating more frequently within-population. Between-population matings were most likely between apple males and hawthorn females, which could facilitate asymmetric gene flow. Asymmetric sexual isolation was also found between two more deeply divergent sibling species in the *R. pomonella* species complex (Yee and Goughnour 2012); thus, asymmetric sexual isolation may persist beyond early stages of divergence. Early in the divergence process, reproductive barriers are often asymmetric, potentially because divergent selection can act unevenly on each population (Lackey and Boughman 2017, Tadeo et al. 2018, Ribardiere et al. 2019, Davis et al. 2021). Gene flow allowed by asymmetric isolation can limit further divergence and halt or reverse the speciation process, especially if asymmetric isolation persists in later stages of divergence (Arnold et al. 1996, Servedio and Kirkpatrick 1997, Chunco et al. 2007). However, between more distantly related species in the *Rhagoletis* genus, sexual isolation is complete and symmetric (Hood et al. 2012), suggesting that asymmetries in sexual isolation may diminish as divergence proceeds.

While environmental differences in rearing conditions did not change the strength of sexual isolation between populations, the frequency and duration of mating interactions were sensitive to rearing temperature. Flies reared under our simulated warming temperature regimes copulated less frequently than flies reared under control conditions. Fewer opportunities for reproductive success in warming-reared flies could limit population growth rates given that multiple matings increase fertilization success in this system (Opp and Prokopy 1986). Additionally, warming-reared flies copulated longer in between-population pairs than control-reared flies, which may increase reproductive success of between-population matings. Thus, while warmer temperatures may not weaken sexual isolation, altered mating interactions under warming conditions may affect population maintenance and gene flow.

In this study, we provide evidence of a new dimension of reproductive isolation between recently diverged populations of *R. pomonella*. Members of the *R. pomonella* species complex have undergone a rapid adaptive radiation primarily due to divergent ecological adaptation (Bush 1966, Berlocher 2000, Powell et al. 2013). However, reproductive isolation is incomplete between recently diverged populations in this complex (Powell et al. 2013, Arcella et al. 2015, Inskeep et al. 2021). Thus, ecological divergence alone may be insufficient to complete speciation (e.g., Nosil et al. 2009). Sexual isolation may play an important role in reducing gene flow to an extent that facilitates further divergence and potential speciation. This study emphasizes the importance of understanding the strength and evolution of reproductive barriers that evolve after initial divergence and the role of these barriers in population divergence.

References

Aluja, M., N. Lozada, J. Pinero, A. Birke, V. Hernandez-Ortiz, and F. Diaz-Fleischer. 2001. Basic behavior of *Rhagoletis turpiniae* (Diptera: Tephritidae) with comparative notes on the sexual behavior of *Rhagoletis pomonella* and *Rhagoletis zoqui*. Annals of the Entomological Society of America **94**:268-274.

Arcella, T., G. R. Hood, T. H. Powell, S. B. Sim, W. L. Yee, D. Schwarz, S. P. Egan, R. B. Goughnour, J. J. Smith, and J. L. Feder. 2015. Hybridization and the spread of the apple maggot fly, Rhagoletis pomonella (Diptera: Tephritidae), in the northwestern United States. Evol Appl **8**:834-846.

Arnold, S. J., P. A. Verrell, and S. G. Tilley. 1996. The evolution of asymmetry in sexual isolation: a model and a test case. Evolution **50**:1024-1033.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models Usinglme4. Journal of Statistical Software **67**.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society B **57**:289-300.

Berlocher, S. H. 2000. Radiation and Divergence in the Rhagoletis Pomonella Species Group: Inferences from Allozymes. Evolution **54**.

Berlocher, S. H., and J. L. Feder. 2002. Sympatric speciation phytophagous insects: moving beyond controversy? Annual Review of Entomology **47**:773-815.

Boughman, J. W. 2002. How sensory drive can promote speciation. Trends in Ecology & Evolution **17**:571-577.

Boughman, J. W., and R. Svanback. 2017. Synergistic selection between ecological niche and mate preference primes diversification. Evolution **71**:6-22.

Bush, G. L. 1966. The taxonomy, cytology and evolution of the genus *Rhagoletis* in North America (Diptera:Tiphritidae). Museum of Comparative Zoology, Cambridge, Massachusetts.

Butlin, R. K., and C. M. Smadja. 2018. Coupling, Reinforcement, and Speciation. Am Nat **191**:155-172.

Chunco, A. J., J. S. McKinnon, and M. R. Servedio. 2007. Microhabitat variation and sexual selection can maintain male color polymorphisms. Evolution **61**:2504-2515.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Inc., Sunderland, Massachusetts.

Dambroski, H. R., and J. L. Feder. 2007. Host plant and latitude-related diapause variation in Rhagoletis pomonella: a test for multifaceted life history adaptation on different stages of diapause development. J Evol Biol **20**:2101-2112.

Davis, J. S., M. J. Pearcy, J. Y. Yew, and L. C. Moyle. 2021. A shift to shorter cuticular hydrocarbons accompanies sexual isolation among Drosophila americana group populations. Evol Lett **5**:521-540.

Dieckmann, U., and M. Doebeli. 2004. Adaptive dynamics of speciation: sexual populations.*in* U. Dieckmann, M. Doebeli, J. A. J. Metz, and D. Tautz, editors. Adaptive Speciation. Cambridge University Press, Cambridge.

Dopman, E. B., P. S. Robbins, and A. Seaman. 2010. Components of reproductive isolation between North American pheromone strains of the European corn borer. Evolution **64**:881-902.

Feder, J. L. 1995. The effects of parasitoids on sympatric host races of *Rhagoletis pomonella* (Diptera:Tephritidae). Ecology **76**:801-813.

Feder, J. L., C. A. Chilcote, and G. L. Bush. 1988. Genetic differentiation between sympatric host races of the apple maggot fly *Rhagoletis pomonella*. Nature **336**:61-64.

Feder, J. L., S. B. Opp, B. Wlazlo, K. Reynolds, W. Go, and S. Spisak. 1994. Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. Proceedings of the Academy of Natural Sciences of the United States of America **91**:7990-7994.

Feder, J. L., T. H. Q. Powell, K. Filchak, and B. Leung. 2010. The diapause response of Rhagoletis pomonella to varying environmental conditions and its significance for geographic and host plant-related adaptation. Entomologia Experimentalis Et Applicata **136**:31-44.

Fisher, H. S., B. B. Wong, and G. G. Rosenthal. 2006. Alteration of the chemical environment disrupts communication in a freshwater fish. Proc Biol Sci **273**:1187-1193.

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. Molecular Ecology **23**:4074-4088.

Grant, B. R., and P. R. Grant. 2008. Fission and fusion of Darwin's finches populations. Philos Trans R Soc Lond B Biol Sci **363**:2821-2829.

Heath, D., C. M. Bettles, and D. Roff. 2010. Environmental factors associated with reproductive barrier breakdown in sympatric trout populations on Vancouver Island. Evol Appl **3**:77-90.

Hood, G. R., S. P. Egan, and J. L. Feder. 2012. Evidence for sexual isolation as a prezygotic barrier to gene flow between morphologically divergent species ofRhagoletisfruit flies. Ecological Entomology **37**:521-528.

Hood, G. R., T. H. Q. Powell, M. M. Doellman, S. B. Sim, M. Glover, W. L. Yee, R. B. Goughnour, M. Mattsson, D. Schwarz, and J. L. Feder. 2020. Rapid and repeatable host plant shifts drive reproductive isolation following a recent human-mediated introduction of the apple maggot fly, Rhagoletis pomonella. Evolution **74**:156-168.

Inskeep, K. A., M. M. Doellman, T. H. Q. Powell, S. H. Berlocher, N. R. Siefert, G. R. Hood, G. J. Ragland, P. J. Meyers, and J. L. Feder. 2021. Divergent diapause life history timing drives both allochronic speciation and reticulate hybridization in an adaptive radiation of *Rhagoletis* flies. Molecular Ecology.

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

Kaneshiro, K. Y. 1980. Sexual isolation, speciation and the direction of evolution. Evolution **34**:437-444.

Kautt, A. F., C. F. Kratochwil, A. Nater, G. Machado-Schiaffino, M. Olave, F. Henning, J. Torres-Dowdall, A. Harer, C. D. Hulsey, P. Franchini, M. Pippel, E. W. Myers, and A. Meyer. 2020. Contrasting signatures of genomic divergence during sympatric speciation. Nature **588**:106-111.

Kitano, J., S. Mori, and C. L. Peichel. 2007. Phenotypic divergence and reproductive isolation between sympatric forms of Japanese threespine sticklebacks. Biological Journal of the Linnean Society **91**:671-685.

Kuwajima, M., N. Kobayashi, T. Katoh, and H. Katakura. 2010. Detection of ecological hybrid inviability in a pair of sympatric phytophagous ladybird beetles (Henosepilachna spp.). Entomologia Experimentalis Et Applicata **134**:280-286.

Lackey, A. C., and J. W. Boughman. 2017. Evolution of reproductive isolation in stickleback fish. Evolution **71**:357-372.

Lackey, A. C. R., and J. W. Boughman. 2013. Loss of sexual isolation in a hybridizing stickleback species pair. Current Zoology **59**:591-603.

Lande, R., and M. Kirkpatrick. 1988. Ecological speciation by sexual selection. Journal of Theoretical Biology **133**:85-98.

Lenth, R. V. 2021. emmeans: Estimates marginal means, aka least-squares means. R package version 1.6.0. https://CRAN.R-project.org/package=emmeans

Linn, C., J. L. Feder, S. Nojima, H. R. Dambroski, S. H. Berlocher, and W. Roelofs. 2003. Fruit odor discrimination and sympatric host race formation in *Rhagoletis*. Proceedings of the Academy of Natural Sciences of the United States of America **100**:11490-11493.

Linn, C., S. Nojima, and W. Roelofs. 2005. Antagonist effects of non-host fruit volaties on discrimination of host fruit by *Rhagoletis* flies infesting apple (*Malus pumila*), hawthorn (*Crataegus* spp.), and flowering dogwood (*Cornus florida*). Entomologia Experimentalis Et Applicata **114**:97-105.

Linn, C. E., H. R. Dambroski, J. L. Feder, S. H. Berlocher, S. Nojima, and W. Roelofs. 2004. Postzygotic isolating factor in sympatric speciation in *Rhagoletis* flies: Reduced response of hybrids to parental host-fruit odors. Proceedings of the Academy of Natural Sciences of the United States of America **101**:17753-17758.

Linn, C. E., Jr., W. L. Yee, S. B. Sim, D. H. Cha, T. H. Powell, R. B. Goughnour, and J. L. Feder. 2012. Behavioral evidence for fruit odor discrimination and sympatric host races of Rhagoletis pomonella flies in the Western United States. Evolution **66**:3632-3641.

Lowry, D. B., J. L. Modliszewski, K. M. Wright, C. A. Wu, and J. H. Willis. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. Philos Trans R Soc Lond B Biol Sci **363**:3009-3021.

Lyons-Sobaski, S., and S. H. Berlocher. 2009. Life history phenology differences between southern and northern populations of the apple maggot fly, *Rhagoletis pomonella*. Entomologia Experimentalis Et Applicata **130**:149-159.

Maan, M. E., and O. Seehausen. 2011. Ecology, sexual selection and speciation. Ecol Lett **14**:591-602.

Marques, D. A., J. I. Meier, and O. Seehausen. 2019. A combinatorial view on speciation and adaptive radiation. Trends in Ecology and Evolution **34**:531-544.

Mattsson, M., G. R. Hood, W. L. Yee, M. M. Doellman, D. J. Bruzzese, R. B. Goughnour, A. L. Driscoe, S. Van Dexter, C. Tait, M. M. Glover, P. Meyers, L. A. Ruedas, and J. L. Feder. 2021. Recursive adaptation in action: allochronis isolation and divergence of host-associated populations of the apple maggot fly, *Rhagoletis pomonella*, following its recent introduction to the western USA. Entomologia Experimentalis Et Applicata:1-16.

Maxwell, C. W., and E. C. Parsons. 1968. The recapture of marked apple maggot flies in several orchards from one release point. Oecologia **61**:1157-1159.

McNett, G. D., and R. B. Cocroft. 2008. Host shifts favor vibrational signal divergence in Enchenopa binotata treehoppers. Behavioral Ecology **19**:650-656.

Mendelson, T. C., V. E. Imhoff, and J. J. Venditti. 2007. The accumulation of reproductive barriers during speciation: Postmating barriers in two behaviorally isolated species of darters (percidae : etheostoma). Evolution **61**:2596-2606.

Mendelson, T. C., M. D. Martin, and S. M. Flaxman. 2014. Mutation-order divergence by sexual selection: diversification of sexual signals in similar environments as a first step in speciation. Ecology Letters **17**:1053-1066.

Merrill, R. M., Z. Gompert, L. M. Dembeck, M. R. Kronforst, W. O. McMillan, and C. D. Jiggins. 2011. Mate preference across the speciation continuum in a clade of mimetic butterflies. Evolution **65**:1489-1500.

Michel, A. P., S. Sim, T. H. Powell, M. S. Taylor, P. Nosil, and J. L. Feder. 2010. Widespread genomic divergence during sympatric speciation. Proc Natl Acad Sci U S A **107**:9724-9729.

Neilson, W. T. A., and J. W. McAllan. 1964. Artificial diets for the apple maggot, *Rhagoletis pomonella*. I. Mass rearing on certain diets. Journal of Economic Entomology **57**:333-335.

Nosil, P. 2012. Ecological Speciation. Oxford University Press, Oxford.

Nosil, P., and J. L. Feder. 2012. Genomic divergence during speciation: causes and consequences. Philos Trans R Soc Lond B Biol Sci **367**:332-342.

Nosil, P., J. L. Feder, S. M. Flaxman, and Z. Gompert. 2017. Tipping points in the dynamics of speciation. Nat Ecol Evol **1**:1.

Nosil, P., L. J. Harmon, and O. Seehausen. 2009. Ecological explanations for (incomplete) speciation. Trends Ecol Evol **24**:145-156.

Nosil, P., T. H. Vines, and D. J. Funk. 2005. Perspective: reproductive isolation cause by natural selection against immigrants from divergent habitats. Evolution **59**:705-719.

Opp, S. B., and R. J. Prokopy. 1986. Veriation in laboratory oviposition by *Rhagoletis pomonella* (Diptera: Tephritidae) in relation to mating success. Annals of the Entomological Society of America **79**:705-710.

Powell, T. H., A. A. Forbes, G. R. Hood, and J. L. Feder. 2014. Ecological adaptation and reproductive isolation in sympatry: genetic and phenotypic evidence for native host races of Rhagoletis pomonella. Mol Ecol **23**:688-704.

Powell, T. H., G. R. Hood, M. O. Murphy, J. S. Heilveil, S. H. Berlocher, P. Nosil, and J. L. Feder. 2013. Genetic divergence along the speciation continuum: the transition from host race to species in rhagoletis (Diptera: tephritidae). Evolution **67**:2561-2576.

Powell, T. H. Q., A. D. Nguyen, Q. Xia, J. L. Feder, G. J. Ragland, and D. A. Hahn. 2020. A rapidly evolved shift in life-history timing during ecological speciation is driven by the transition between developmental phases. Journal of Evolutionary Biology **33**:1371-1386.

Prokopy, R. J. 1976. Feeding, mating and oviposition activities of *Rhagoletis fausta* flies in nature. Annals of the Entomological Society of America **69**:899-904.

Pryor, S. C., R. J. Barthelmie, and J. T. Schoof. 2013. High-resolution projections of climate-related risks for the Midwestern USA. Climate Research **56**:61-79.

R Team. 2021. R: A language and environment for statistical computer. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

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

Ribardiere, A., E. Pabion, J. Coudret, C. Daguin-Thiebaut, C. Houbin, S. Loisel, S. Henry, and T. Broquet. 2019. Sexual isolation with and without ecological isolation in marine isopods Jaera albifrons and J. praehirsuta. J Evol Biol **34**:33-48.

Rice, W. R., and E. E. Hostert. 1993. Laboratory experiments on speciation: what have we learned in 40 years? Evolution **47**:1637-1653.

Ritchie, M. G. 2007. Sexual Selection and Speciation. Annual Review of Ecology, Evolution, and Systematics **38**:79-102.

Rull, J., M. Aluja, and J. L. Feder. 2010. Evolution of intrinsic reproductive isolation among four North American populations of *Rhagoletis pomonella* (Dipter: Tephritidae). Biological Journal of the Linnean Society **100**:213-223.

Rundle, H. D., and P. Nosil. 2005. Ecological speciation. Ecology Letters **8**:336-352.

Rundle, H. D., and L. Rowe. 2018. The contribution of sexual selection to ecological and mutation-order speciation. Evolution **72**:2571-2575.

Safran, R. J., E. S. C. Scordato, L. B. Symes, R. L. Rodriguez, and T. C. Mendelson. 2013. Contributions of natural and sexual selection to the evolution of premating reproductive isolation: a research agenda. Trends in Ecology & Evolution **28**:643-650.

Schemske, D. W. 2010. Adaptation and the origin of species. Am Nat **176 Suppl 1**:S4-S25.

Schilling, M. P., S. P. Mullen, M. Kronforst, R. J. Safran, P. Nosil, J. L. Feder, Z. Gompert, and S. M. Flaxman. 2018. Transitions from Single- to Multi-Locus Processes during Speciation with Gene Flow. Genes (Basel) **9**.

Schluter, D. 2000. The Ecology of Adaptive Radiation. Oxford University Press, Oxford.

Schluter, D. 2001. Ecology and the origin of species. Trends in Ecology & Evolution **16**:372-379.

Schwarz, D., and B. A. McPherson. 2007. When ecological isolation breaks down: sexual isolation is an incomplete barriers to hybridization between *Rhagoletis* species. Evolutionary Ecology Research **9**:829-841.

Scordato, E. S., L. B. Symes, T. C. Mendelson, and R. J. Safran. 2014. The role of ecology in speciation by sexual selection: a systematic empirical review. J Hered **105 Suppl 1**:782-794.

Seehausen, O., J. J. M. v. Alphen, and F. Witte. 1997. Cichlid Fish Diversity Threatened by Eutrophication That Curbs Sexual Selection. Science **277**:1808-1811.

Seehausen, O., G. Takimoto, D. Roy, and J. Jokela. 2008. Speciation reversal and biodiversity dynamics with hybridization in changing environments. Mol Ecol **17**:30-44.

Servedio, M. R., and J. W. Boughman. 2017. The Role of Sexual Selection in Local Adaptation and Speciation. Pages 85-109 *in* D. J. Futuyma, editor. Annual Review of Ecology, Evolution, and Systematics, Vol 48.

Servedio, M. R., and M. Kirkpatrick. 1997. The effects of gene flow on reinforcement. Evolution **51**:1764-1772.

Servedio, M. R., and M. A. F. Noor. 2003. The Role of Reinforcement in Speciation: Theory and Data. Annual Review of Ecology, Evolution, and Systematics **34**:339-364.

Servedio, M. R., G. S. Van Doorn, M. Kopp, A. M. Frame, and P. Nosil. 2011. Magic traits in speciation: 'magic' but not rare? Trends Ecol Evol **26**:389-397.

Smadja, C. M., and R. K. Butlin. 2011. A framework for comparing processes of speciation in the presence of gene flow. Mol Ecol **20**:5123-5140.

Smith, D. C. 1988. Genetics and reproductive isolation of *Rhagoletis* flies. University of Illinois at Urbana-Champaign, Urbana, Illinois.

Smith, D. C., and R. J. Prokopy. 1982. Mating behavior of *Rhagoletis mendax* (Diptera: Tephritidae) flies in nature. Annals of the Entomological Society of America **75**:388-392.

Sobel, J. M., and G. F. Chen. 2014. Unification of methods for estimating the strength of reproductive isolation. Evolution **68**:1511-1522.

Tadeo, E., M. Aluja, and J. Rull. 2018. Precopulatory mating and postzygotic isolation between two walnut-infesting species ofRhagoletisfrom Mexican highlands. Entomologia Experimentalis Et Applicata **166**:713-723.

Takami, Y., N. Nagata, M. Sasabe, and T. Sota. 2007. Asymmetry in reproductive isolation and its effect on directional mitochondrial introgression in the parapatric ground beetles Carabus yamato and C. albrechti. Population Ecology **49**:337-346.

Thibert-Plante, X., and A. P. Hendry. 2011. Factors influencing progress toward sympatric speciation. Journal of Evolutionary Biology **24**:2186-2196.

Tiffin, P., M. S. Olson, and L. C. Moyle. 2001. Asymmetrical crossing barriers in angiosperms. Proc Biol Sci **268**:861-867.

Turbek, S. P., M. Browne, A. S. Di Giacomo, C. Kopuchian, W. M. Hochachka, C. Estalles, D. A. Lijtmaer, P. L. Tubaro, L. F. Silveira, I. J. Lovette, R. J. Safran, S. A. Taylor, and L. Campagna. 2021. Rapid speciation via the evolution of pre-mating isolation in the Ibera Seedeater. Science **371**.

Turelli, M., and L. C. Moyle. 2007. Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. Genetics **176**:1059-1088.

van Doorn, G. S., P. Edelaar, and F. J. Weissing. 2009. On the origin of species by natural and sexual selection. Science **326**:1704-1707.

Verhoeven, K. J. F., K. L. Simonsen, and L. M. McIntyre. 2005. Implementing false discovery rate control: increasing your power. Oikos **108**:643-647.

Vonlanthen, P., D. Bittner, A. G. Hudson, K. A. Young, R. Muller, B. Lundsgaard-Hansen, D. Roy, S. Di Piazza, C. R. Largiader, and O. Seehausen. 2012. Eutrophication causes speciation reversal in whitefish adaptive radiations. Nature **482**:357-362.

Wade, M. J., N. W. Chang, and M. McNaughton. 1995. Incipient speciation in the flour beetle, *Tribolium confusum*: premating isolation between natural populations. Heredity **75**:453-459.

Wagner, C. E., L. J. Harmon, and O. Seehausen. 2012. Ecological opportunity and sexual selection together predict adaptive radiation. Nature **487**:366-369.

Walsh, B. D. 1861. On phytophagic varieties and phytophagic species.

Ward, J. L., and M. J. Blum. 2012. Exposure to an environmental estrogen breaks down sexual isolation between native and invasive species. Evol Appl **5**:901-912.

Weissing, F. J., P. Edelaar, and G. S. van Doorn. 2011. Adaptive speciation theory: a conceptual review. Behavioral Ecology and Sociobiology **65**:461-480.

Yeaman, S., and M. C. Whitlock. 2011. The genetic architecture of adaptation under migration-selection balance. Evolution **65**:1897-1911.

Yee, W. L., and R. B. Goughnour. 2012. Mating frequencies and production of hybrids by Rhagoletis pomonella and Rhagoletis zephyria (Diptera: Tephritidae) in the laboratory. The Canadian Entomologist **143**:82-90.

Zhang, L., X. Thibert-Plante, J. Ripa, R. Svanback, and A. Brannstrom. 2019. Biodiversity loss through speciation collapse: Mechanisms, warning signals, and possible rescue. Evolution **73**:1504-1516.

Supporting Information

<u>Supplemental methods text</u>:

In each weekly program, temperatures ramped linearly through four set points: midpoint temperature at sunrise, maximum temperature at the time halfway between sunrise and sunset, midpoint temperature at sunset, and minimum temperature at the time halfway between sunset and sunrise. The timing and length of light:dark cycles were set by sunrise and sunset times for the last day in each week of 2016 at the Watseka station. When median weekly temperatures would have dropped below 6°C in each temperature regime, we switched environmental chambers to a winter program with lights off and 2.5°C minimum, 3.0°C midpoint, and 3.5°C maximum set points. When median weekly temperatures would have risen above 6°C, we switched environmental chambers to resume Control and Warming regimes based on 10-year weekly temperature averages and light:dark cycles. Given differences in when

Control and Warming median temperatures would drop below and rise above 6°C, winter length differed between temperature regimes: 20 weeks, November 12 to April 1, for Control; 16 weeks, November 19 to March 11 for Warming.

Supplemental Table 1: For each of three prezygotic reproductive barriers, we provide values for the individual barrier strength, 95% confidence interval width, upper and lower bounds of the individual strength given the confidence interval, and the sequential strength. The sequential strength is calculated from its individual strength and the amount of gene flow allowed by earlier-acting barriers.

barrier	individual strength	95% CI	upper	lower	sequential strength
temporal	0.4363	0.1234	0.5597	0.3128	0.4363
habitat	0.8746	0.0421	0.9167	0.8325	0.4931
sexual	0.1461	0.0614	0.2075	0.0847	0.0103