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

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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 generations) 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 tested how warmer temperatures predicted under climate change could alter sexual isolation and found that sexual isolation was markedly asymmetric between the sexes of each population when flies were reared under warmer temperatures. Our findings provide a window into the early divergence process and the role of sexual isolation after initial ecological divergence, in addition to examining how environmental conditions 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). Moreover, the coupling of multiple barrier traits may drive rapid transitions along the speciation continuum, promoting strong reproductive isolation and widespread genomic differentiation (Barton and De Cara 2009, Flaxman et al. 2014, Kunerth et al. 2022). Thus, understanding how multifaceted reproductive isolation develops along the speciation continuum following initial ecological divergence has important implications for the tempo of diversification.

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, Zhang et al. 2022). 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, the apple maggot fly, *Rhagoletis pomonella*, to evaluate how multifaceted reproductive isolation may evolve, particularly early in divergence. *Rhagoletis pomonella* is a textbook case of ecological speciation-in-action (Dres and Mallet 2002, Coyne & Orr 2004, Futuyma 2013). A population of these flies shifted from infesting the fruit of native downy hawthorn (*Crataegus mollis*) to introduced apple (*Malus pumila*) during the mid-19th century (Walsh 1861, Bush 1966) and divergent adaptation to these two host plants in the subsequent ~170 generations has led to substantial but incomplete reproductive isolation between the two host-associated populations of *R. pomonella* (Feder et al. 1988, 1994; Michel et al. 2010). The resulting consistent allele frequency differentiation between sympatric apple and hawthorn-infesting population pairs support the position of the derived apple fly at the hypothesized “host race” stage of ecological speciation in phytophagous insects (Berlocher and Feder 2002, Dres and Mallet 2002, Powell et al. 2013; 2022). The primary axes of divergent host plant adaptation driving reproductive isolation in this system are chemosensory adaptation to host fruit volatiles, which are the major cues for mating aggregation (Linn et al. 2003) and diapause-mediated life history timing corresponding to differences in fruiting phenology of the host plants (Filchak et al. 2000, Feder et al. 2010). These traits act as prezygotic barriers to gene flow by restricting inter-host mating opportunities both spatially and temporally (Feder et al. 1994; Forbes et al. 2005) and as post-zygotic barriers via maladaptive phenotypes for both traits in F1 hybrids (Linn et al. 2004, Dambroski & Feder 2007). The divergence in these traits and their role in this incipient speciation system have been well characterized at the phenotypic, physiological, genetic, and, in the case of diapause, genomic levels (e.g., Dambroski et al. 2005; Forbes et al. 2005; Olsson et al. 2006; Dambroski and Feder 2007, Tait et al. 2016; 2021, Powell et al. 2020; Dowle et al. 2020; Calvert et al. 2022).

While habitat and temporal isolation strongly limit gene flow, apple and hawthorn flies can still encounter each other, and mark recapture estimates indicate gross migration of ~6% in sympatry (Feder et al. 1994). Whether this incomplete state of speciation is a transient phase in a still-progressing process or a long-term stalemate between divergent selection and migration remains unclear, but additional reproductive barriers may be necessary for additional divergence to accumulate (Ragland et al. 2015). Previous research found complete or nearly complete sexual isolation between highly divergent species pairs in the *Rhagoletis* genus (Hood et al. 2012), indicating that this barrier commonly contributes to the speciation process in these flies. In very recently diverged populations of *R. pomonella,* however, questions remain as to the presence and strength of sexual isolation as well as the potential forces that might underlie this barrier.

Given the potential potency of sexual isolation acting in concert with known ecological isolation to drive rapid divergence, we made a novel extension of this classic 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. Lastly, we tested whether rearing fly pupae under temperature regimes that mimic climate change predictions in the next 50-100 years affected mating interactions with consequences for the strength of sexual isolation as on-going speciation may be altered by anthropogenic change if reproductive barriers are environmentally sensitive.

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. This sympatric population pair has been one of the most extensively studied in the *R. pomonella* species complex over the last four decades and provided one of the first population genetic confirmations of incipient sympatric speciation (McPheron et al. 1988). Since then, the apple and hawthorn populations in Urbana, IL have contributed to our understanding of the divergent adaption of chemosensory behavior (e.g., Linn et al. 2003; 2004; 2005, Dambroski et al. 2005, Olsson et al. 2006) and diapause-mediated phenology (e.g., Dambroski & Feder 2007, Meyers et al. 2016, Powell et al. 2020, Dowle et al. 2020) as well as the population genomics of differentiation (e.g., Feder et al. 2003; Schwarz et al. 2009; Michel et al. 2010, Ragland et al. 2017, Doellman et al. 2018; 2019, Dowle et al. 2020, Calvert et al. 2022). Thus, the patterns of ecological divergence and genetic relationship between the apple and hawthorn flies at this site are well-established, providing a robust foundation for testing for the presence of additional axes of divergence and reproductive isolation. 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. The flies used in this experiment were derived from a large-scale climate change simulation study testing for the effect of temperature on pupal developmental timing. 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). In our study, copulations occurred throughout the mating tent and rarely directly on the fruit. 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. 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).

Data analysis

Sexual isolation

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

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

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) 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):

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

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 also calculated sexual isolation separately by rearing temperature (Control or Warming) to assess environmental effects on the total strength of SI and the contribution of each sex from each population. For Control temperatures, the sample sizes were 69 copulations total with 28 copulations with Apple females, 37 copulations with Apple males, 41 copulations with Hawthorn females, and 32 copulations with Hawthorn males. For Warming temperatures, the sample sizes were 58 copulations total with 25 copulations with Apple females, 33 copulations with Apple males, 33 copulations with Hawthorn females, and 25 copulations with Hawthorn males.

Comparing prezygotic isolating barriers

To place the strength of sexual isolation in context of 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 (unpublished data). 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):

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

Results

Sexual isolation

Sexual isolation between Apple and Hawthorn flies was significantly greater than expectations of random mating, where isolation is zero, in both Control and Warming rearing treatments, (Control: SI = 0.191 [95%CI: 0.284 - 0.093], Warming: SI = 0.116 [95%CI: 0.199 - 0.034], Figure 1, Supplemental Table 1). Given the overlap of 95% confidence intervals, the strength of sexual isolation did not differ between temperature treatments (Figure 1, Supplemental Table 1). However, the pattern of the contributions to total sexual isolation from each sex of each population differed between temperature treatments. In the Control treatment, all flies mated within population more than between population, and contributions to sexual isolation from each sex from each population were significantly greater than expectations of random mating (Figure 2A). In the Warming treatment, in contrast, Apple females and Hawthorn males mated within population more than between population with measures of sexual isolation significantly greater than 0, while Apple males and Hawthorn females mated randomly within and between population (Figure 2B, Supplemental Table 1, Supplemental Figure 1).

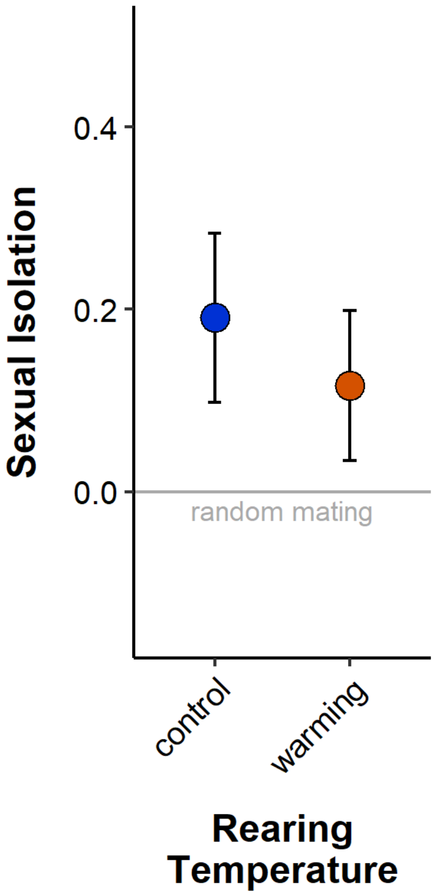

Figure 1. Total sexual isolation for flies reared under Control (blue) and Warming (orange) temperatures. Circles are point values with 95% CIs. The horizontal grey line at 0 indicates random mating, and positive values indicate greater mating within populations than between. Values for barrier strengths and 95% CIs are provided in Supplemental Table 1.

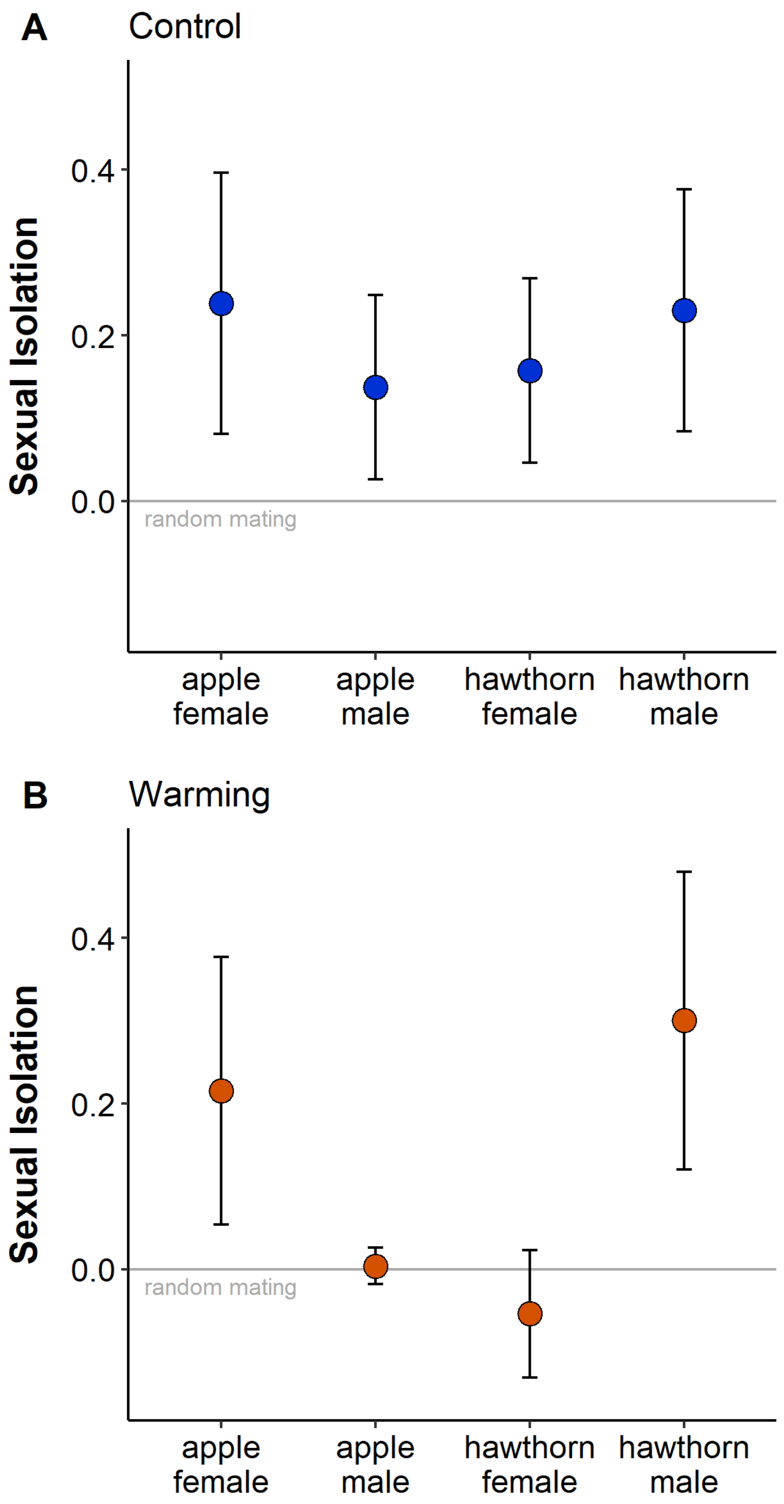

Figure 2. Contributions of each sex from each population to sexual isolation for flies reared under Control (blue) and Warming (orange) temperatures. Circles are point values with 95% CIs. The horizontal grey

line at 0 indicates random mating, and positive values indicate greater mating within populations than between. Values for barrier strengths and 95% CIs are provided in Supplemental Table 1.

Comparing prezygotic isolating barriers

Individual strengths of isolating barriers estimate the proportion of gene flow limited by each barrier if acting alone. We compared total sexual isolation from Control rearing conditions calculated in this study to measures of temporal isolation from our unpublished data on eclosion timing and habitat isolation from previously published work on attraction preference to host fruit volatiles. Temporal isolation was moderate in strength (RI = 0.44, 95% CI: 0.31 – 0.56). Habitat isolation 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 estimate the proportion of gene flow limited by each barrier given the gene flow allowed by earlier-acting barriers. Together, temporal and habitat isolation were estimated to limit 93% of potential gene flow (RI = 0.93). Sexual isolation would strengthen total RI to 0.94, a 14% change in gene flow allowed.

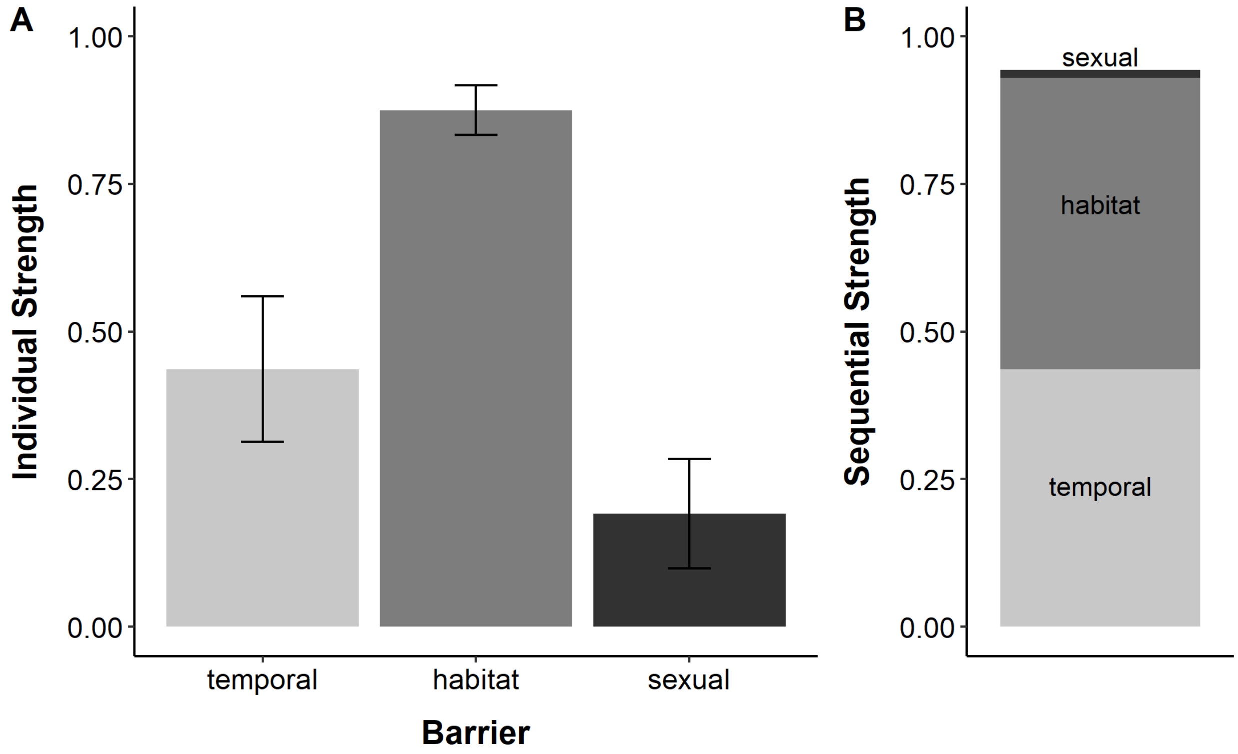

Figure 3. (A) Individual and (B) sequential strengths of three prezygotic barriers. Error bars in A are 95% confidence intervals. Values for barrier strengths and 95% CIs are provided in Supplemental Table 2, and sexual isolation calculations use mating interactions for flies reared under Control temperatures.

Discussion

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). Moreover, 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, sexual isolation, that has evolved within ~170 generations. We found (1) that the strength of sexual isolation was significantly greater than expectations of random mating, and (2) sexual isolation was symmetric between the sexes of each population for flies reared under control conditions but asymmetric for flies were reared under warmer temperatures.

Between apple and hawthorn flies, we provide evidence that sexual isolation could limit approximately 19% 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). Thus, sexual isolation may have evolved rapidly early in divergence. 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). Notably, 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). 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). Yet, it is currently unknown whether sexual isolation evolves in association with host adaptation or independently.

In ecological speciation, barriers under direct divergent selection evolve first, and subsequent barriers can evolve as a by-product of divergent adaptation or independently (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). Sexual isolation can evolve as a by-product of ecological adaptation to different host fruits if traits under divergent ecological selection are also mating traits (Servedio et al. 2011). Sexual isolation may also 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. Lastly, sexual isolation could 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.

When isolation is symmetric, gene flow is limited similarly by both populations, and this bi-directional reduction in gene flow yields more stable isolation. Asymmetric isolation, in contrast, allows gene flow more in one direction than another between populations and 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). In this study, flies reared under control temperatures, showed no asymmetry in sexual isolation; each sex from each population mated more within population than between. Thus, sexual isolation can limit gene flow similarly between both populations. Under warming conditions, however, sexual isolation was asymmetric between the sexes of each population. Apple males and hawthorn females mated randomly while apple females and hawthorn males mated more within population than between. Though warmer rearing temperatures did not change the overall strength of sexual isolation between populations, asymmetric contributions to total sexual isolation under warming conditions could facilitate asymmetric gene flow. 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). Potentially, plasticity in mating traits and preferences that determine the likelihood of mating within or between population could shape the strength and/or symmetric of sexual isolation (e.g., Jin et al. 2022).

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.

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Supporting Information

Supplemental methods text:

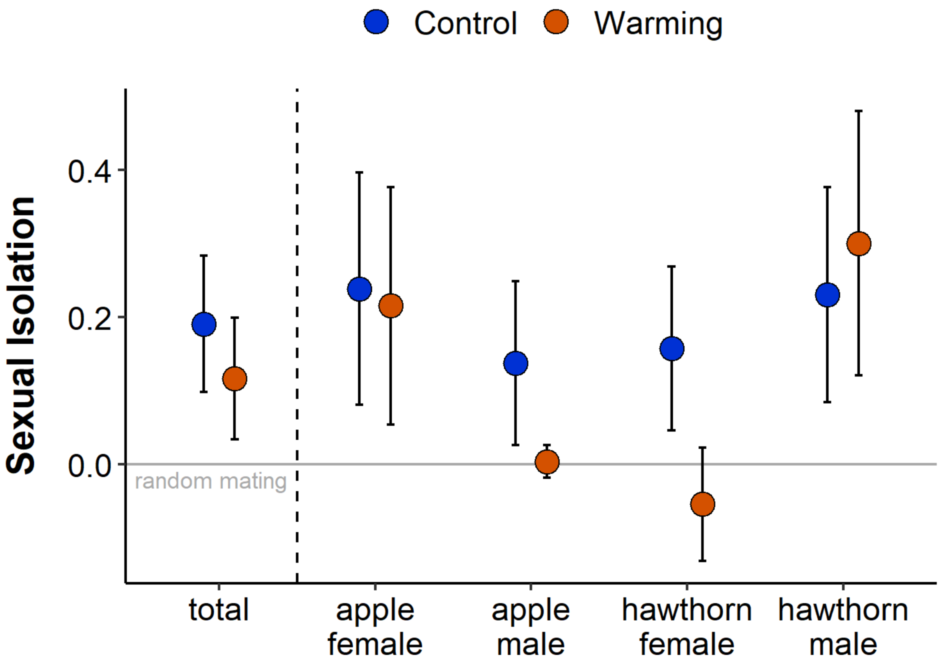
In each weekly program, temperatures ramped linearly through four set points: midpoint temperature at sunrise, maximum temperature at the time halfway between sunrise and sunset, midpoint temperature at sunset, and minimum temperature at the time halfway between sunset and sunrise. The timing and length 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: Measures of sexual isolation with 95% confidence interval upper and lower limits for total sexual isolation as well as contributions from each sex of each population for flies reared in Control and Warming temperature treatments.

	Control treatment			Warming treatment		
	Sexual isolation	95% CI upper limit	95% CI lower limit	Sexual isolation	95% CI upper limit	95% CI lower limit
Total	0.191	0.284	0.098	0.116	0.199	0.034
Apple female	0.239	0.397	0.081	0.216	0.377	0.054
Apple male	0.138	0.249	0.027	0.004	0.026	-0.018
Hawthorn female	0.158	0.269	0.046	-0.054	0.023	-0.131
Hawthorn male	0.231	0.376	0.085	0.301	0.480	0.121

Supplemental Table 2: 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. Sexual isolation is based on mating interactions in flies from Control rearing temperatures.

barrier	individual strength	95% CI upper limit	95% CI lower limit	sequential strength
temporal	0.4363	0.5597	0.3128	0.4363
habitat	0.8746	0.9167	0.8325	0.4931
sexual	0.1909	0.2837	0.0982	0.0135



<u>Supplemental Figure 1</u>. Total sexual isolation and contributions of each sex from each population for flies reared under Control (blue) and Warming (orange) temperatures. 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. Values for barrier strengths and 95% CIs are provided in Supplemental Table 1.