Postmating reproductive barriers contribute to the incipient sexual isolation of US and Caribbean *Drosophila melanogaster*

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Abstract

The nascent stages of speciation start with the emergence of sexual isolation. Understanding how reproductive barriers influence this evolutionary process is an ongoing effort. We present here a study of *Drosophila melanogaster* populations from the southeast United States and Caribbean islands undergoing incipient sexual isolation. The existence of premating reproductive barriers have been previously established, but they do not fully account for the degree of isolation present. To assess the influence of postmating barriers, we investigated putative postmating barriers of female remating and egg laying behavior, as well as hatchability of eggs laid and female longevity after mating. While we did not find any effects in female remating or egg laying, we did observe lower hatchability in the central region of our geographical spread as well as shorten female life spans after mating to genetically different males in females originating from the northern- and southernmost locations of those surveyed. These results serve as evidence that long-term consequences after mating such as the fitness of offspring and shortened lifespan have a stronger effect than short-term postmating behaviors.

Running Title:

Postmating barriers in US and Caribbean fruit flies

Key Words

sexual conflict, egg laying, hatchability, remating, sperm toxicity, chase away selection

Introduction

Males and females in sexual mating systems ultimately have different reproductive interests and this interaction of contrasting interests is commonly referred to as sexual conflict (Chapman et al. 2003; Parker 1979). Competitive males are selected to override the mating preferences evolved by females (Holland and Rice 1998). Females consequently evolve resistance to male 'coercion' tactics, and males are then selected by novel or more exaggerated traits - perpetuating an endless evolutionary chase between the sexes (Arbuthnott et al. *2014*; Parker 1979; Civetta and Singh 1995; Rice 1996; Chapman et al. 2003; Arnqvist and Rowe 2005). This phenomenon of conflict in reproductive optima has been experimentally demonstrated to promote an antagonistic male-female coevolution that is the essence of sexual isolation which precedes speciation (Chapman et al. 2003; Parker 1979; Holland and Rice 1998).

The tempo and mode by which new species are formed are influenced by the development of reproductive barriers, which facilitate reproductive isolation. When multiple barriers accumulate, gene flow is reduced between or within a given population, which can give rise to early stages of speciation. These reproductive barriers are traditionally classified as those occurring before fertilization, prezygotic, and those occurring after fertilization, postzygotic (Coyne and Orr 2004). The latter can be further broken down into extrinsic and intrinsic depending on if the barrier interacts with external factors (i.e. environmental, individuals) or internal factors (i.e. genetic incompatibilities) (Seehausen et al., 2014). Speciation involves multiple reproductive barriers of varying effect sizes (Coyne and Orr 2004, Seehausen et al., 2014), and identifying the interactivity and varying strengths of reproductive barriers at play is vital to characterizing the process of speciation.

Flies from the *Drosophila* genus are particularly suited to study the rise and fall of reproductive barriers since the range of study systems span the whole speciation spectrum from completely non-interbreeding species to hybridizing species (Bono and Markow, 2009), as well as species experiencing sexual isolation within itself (Yukilevich and True, 2008b). Empirical studies investigating sexual selection in *D. melanogaster* have been conducted about the evolution of prezygotic isolation - mate choice, male morphology, and courtship behavior (Yukilevich and True 2008a; Hollocher 1997). Is has been suggested

that premating barriers are of greater strength (Cozzolino and Scopece 2008; Coyne and Orr 1989; Ramsey *et al.* 2003; Coyne and Orr 2004). Postzygotic barrier mechanisms have also been known to have an influence in several systems of *Drosophila* flies, but has mostly been studied in hybridizing species such as *D. mojavensis/D. arizonae* (Bono and Markow, 2009) and *D. melanogaster/D. simulans* (Matute et al., 2014). The emergence of postzygotic barriers within a single species has been somewhat less investigated. However, the current knowledge of postmating consequences in *Drosophila melanogaster* suggests that this species is a good model to investigate emerging postmating reproductive barriers.

Male sperm transferred after matting contain accessory gland proteins reduce female remating rates and increase egg laying (Chapman et al. 2003; Wolfner, 1997). Reduced receptivity to remating will also decrease the female opportunity to mate with another male that may result in more fit progeny. Increased egg laying and the trauma from mating reduces female lifespan (Fowler and Partridge 1989). It is even suggested that male sperm is toxic to females (Rice 1996). As a result, females develop resistance to these harmful male traits, and males subsequently evolve new methods to discourage females from mating with other males (Arnqvist and Rowe 2005). It has been suggested that females should be more resistant to males they have co-evolved in with compared to males they have not coevolved with. These effects vary across populations, and ecological context appears to be a factor (Arbuthnott et al. 2014). This rapid, cyclical process termed sexually antagonistic coevolution has been demonstrated in not only Drosophila species (Knowles and Markow 2001) and water striders (Rowe et al. 2002), but also in many other animals. Coevolution by sexual conflict is a strong force behind reproductive isolation, which may lead to speciation in specific circumstances (Martin and Hosken 2003).

Studying areas of where secondary contact where once genetically isolated, allopatric populations interbreed is a powerful approach to understanding the strength and dynamics of male-female postzygotic divergence (Jiggens and Mallet 2000). Of particular interest to this subject are the Caribbean islands and southeastern United States. This area is a secondary contact zone of west African and European *D. melanogaster* populations, which have evolved in allopatry for ~10,000-15,000 years (Capy et al. 1986).

Secondary contact occurred in two waves with west African flies migrating with the transatlantic slave trade 400-500 years ago to the Caribbean islands and the cosmopolitan flies arriving to the east coast US with European colonists <200 years ago (Capy et al., 1986; Duchen et al., 2013). It is known that in this species members of the African Zimbabwe population very rarely mate with individuals outside of this population, which are commonly referred to as 'cosmopolitan' (Hollocher et al. 1997). The contrasting natures of African and cosmopolitan flies are reflected in the Caribbean and southeast US flies respectively with partial mating isolation between west African flies and US cosmopolitan flies (Yukilevich and True 2008a). Previous authors have noted peculiar morphological, behavioral, and pheromonal differences in these North American populations where Caribbean populations display exceptional African-like morphology unlike those from the United States, which have retained cosmopolitan phenotypes from their European predecessors (Yukilevich and True 2008a; Capy et al. 1993; David and Capy 1988). Recent microsatellite evidence also indicates that United States flies are more genetically similar to African flies than are European flies, suggesting that African alleles may have introgressed into North America via the Caribbean islands (Duchen et al., 2013; Yukilevich and True 2008b; Caracristi and Schlotterer 2003; Capy *et al.* 1986) supporting the existence of a southeast US and Caribbean island 'hybrid' zone between African and European populations. Only mating preferences and other premating/prezygotic reproductive barriers have been formally treated in this system (Yukilevich and True 2008a; Yukilevich and True 2008b), and the presence of incipient postmating sexual isolation in Caribbean populations remain unexplored.

We present our study as an effort to better understand the role of postmating reproductive barriers in a system of *Drosophila melanogaster* experiencing incipient sexual isolation. We have investigated the role of remating, female egg laying, hatchability of laid eggs, and female longevity after mating with different males as putative postmating reproductive barriers. These phenotypes are good candidates to investigate the scope of extrinsic and intrinsic postmating reproductive barriers. We measure each of these phenotypes in females from different locations in the southeast US and Caribbean islands and examine them for geographical patterns, which may reveal if and how these barriers affect this system of southeast United States and Caribbean Island *Drosophila melanogaster*.

116

117 **Materials and Methodology**

118 *Fly Lines and Rearing Conditions*

119 For our phenotypic assays, we used 23 isofemale lines of *Drosophila melanogaster* collected in the
120 summer of 2004 and 2005 (Yukilevich and True 2008). Origins are as following (TABLE 1; FIGURE 1):
121 Birmingham, AL (lines 1-1 and 1-2); Selba, AL (lines 2-1 and 2-2); Thomasville, GA (lines 3-1 and 3-2);
122 Meridian, MS (lines 4-1 and 4-2); Tampa Bay, FL (lines 5-1 and 5-2); Sebastian, FL (line 6-1); Freeport,
123 Grand Bahamas-west (lines 7-1 and 7-2); Bullock's Harbor, Berry Islands (lines 8-1 and 8-2); Cockburn
124 Town, San Salvador (lines 9-1 and 9-2); George Town, Exumas (lines 10-1 and 10-2); Mayaguana,
125 Mayaguana (lines 11-1 and 11-2); Port Au Prince, Haiti (lines 12-1 and 12-2). Original line ID numbers
126 are listed in Supplemental Table 1. All flies were maintained at 25 °C in vials on a standard cornmeal diet
127 (recipe available upon request) and entrained under a 12hr light:12hr dark regime.

128

Map Number	Location	Line(s)
1	Birmingham, AL	1-1 and 1-2
2	Selba, AL	2-1 and 2-2
3	Thomasville, GA	3-1 and 3-2
4	Meridian, MS	4-1 and 4-2
5	Tampa Bay, FL	5-1 and 5-2
6	Sebastian, FL	6-1
7	Freeport, Grand Bahamas - West	7-1 and 7-2
8	Bullock's Harbor, Berry Islands	8-1 and 8-2
9	Cockburn Town, San Salvador	9-1 and 9-2
10	George Town, Exumas	10-1 and 10-2
11	Mayaguana, Mayaguana	11-1 and 11-2
12	Port Au Prince, Haiti	12-1 and 12-2

129 Table 1: Corresponding map locations and lines

130

131 *Egg laying, Hatchability, and Remating Rate Assays*

132 Virgin females were collected from all 23 isofemale lines. Male flies up to one day old were collected from
133 two lines (lines 1-2 and 11-1) located at polar ends of our geographical study region. We chose these two
134 lines as sources for male flies based on clinal distance as well as maximal differences between courtship
135 profiles and physical characteristics (Yukilevich and True 2008b) to account for female mate preference
136 which has been previously established (Yukilevich and True 2008a). All flies were collected on light CO2
137 anesthesia and aged for three to four days before entering our assays. We set up a full factorial
138 experiment where females from each of the isofemale lines were crossed with the two lines from which
139 males were collected. Each cross was replicated 15 times.

140

141 All flies were live manipulated using aspirators for the remainder of the phenotypic assays to avoid any
142 physiological and behavioral effects of CO_2 anesthesia (Badre *et al.* 2005). Assays lasted 24 days and
143 were conducted in two stages. The first stage measured female remating rates and egg laying rates over
144 a 10-day period; during the following 14 days, second stage quantified hatchability rates. In the first stage
145 (i.e. first 10 days), females were transferred daily by aspirator into new vials with standard cornmeal fly
146 food and blue food coloring. The dye helped visualize eggs laid by females without causing any variability
147 in their behavior (Bergland 2012). The vials also had 20 uL of a 10% diluted active yeast mixture to
148 stimulate females' reproductive activity. At lights on (i.e. dawn) on the initial day of the first stage,
149 individual females were aspirated into a vial with two males from either one of the two selected male lines
150 for mating. Approximately 90 minutes were allocated for copulation to occur, and all males were
151 discarded immediately after this time period using an aspirator. Females that did not mate on the first day
152 did not continue in the assay. Fecundity assays were conducted daily after the females were transferred
153 into new vials. To assess short-term and long-term receptivity to remating effects, each individual female
154 was introduced to two new males of the same genotype from her initial mating on the fourth and eighth
155 day of the assay (i.e. three and seven days after initial mating). We allowed 90 minutes on both re-mating
156 days for copulations to occur and all males were discarded via aspirator thereafter.

157

158 On the first day of the second stage of the assay in order to ensure the quality of our phenotypic dataset,

159 female identities were checked to confirm correct sexing from when males were discarded from remating
160 days. Incorrectly sexed vials in which the female - instead of the male - were accidentally discarded were
161 not included in later analysis. Remaining vials that passed the first stage of the experiment were
162 monitored daily for fly eclosion. Flies that eclosed were recorded and discarded immediately. Fly eclosion
163 monitoring was terminated when either three consecutive days of zero fly eclosions or 14 days of
164 monitoring was reached - whichever came first. All phenotyping assays during the first and second stages
165 were conducted within the first three hours of lights on (i.e dawn). All flies from the first stage and eclosing
166 vials in the second stage were incubated at a controlled 25 °C with a light timer set for a 12hr light: 12hr
167 dark cycle.
168
169 *Longevity Assays*
170 For our longevity assays, we phenotyped a subset of lines from the 23 isofemale lines that spanned the
171 southeast United States and Caribbean Islands. Female flies used in our longevity assays originated from
172 (arranged from north to south) Selba, Alabama, USA (line 2-2), Thomasville, Georgia, USA (line 3-1),
173 Freeport, Grand Bahamas-west (line 7-2), Bullock's Harbor, Berry Islands (line 8-1), and Port Au Prince,
174 Haiti (line 12-2). Representative 'American' and 'Caribbean' males were derived lines originating from the
175 same male collection lines used in egg laying, hatchability, and remating assays, i.e. Birmingham,
176 Alabama, USA (line 1-2) and Mayaguana, Mayaguana (line 11-1), respectively. 'Homotypic' crosses were
177 defined as male and female both of either American or Caribbean origin. "Heterotypic" crosses were
178 defined as male and female from different origins (i.e. American x Caribbean or Caribbean x American).
179 Male and females from the same origin were assumed to be more related and genetically similar to each
180 other than those from different origins based on previous evidence (Yukilevich and True 2008b).
181
182 Virgins females were collected on light CO2 anesthesia and aged singly in vials for four days. Males were
183 collected in the same manner and aged in groups of five per vial. We performed crosses in two separate
184 rounds, which lasted approximately 70 and 80 days, respectively. In the first round, we crossed female
185 flies from Selba, Alabama, USA and Port Au Prince, Haiti to either our representative 'American' male or
186 'Caribbean' male. There were 50 replicates for each unique cross. Because of the large effect size from

our initial round, in the rest of our lines we had 25 replicates for each type of cross. In each round, aged female flies were placed with five male flies for 48 hours to ensure mating occurred. Male flies were discarded using an aspirator after the mating period. Female flies were then observed on a regular basis five days per week. Dates of deaths were recorded until the end of the 70 or 80-day observational period. The females were transferred to fresh vials every seven days.

Post-mating behavior data analysis

We examined the effects of geographic location on the total number of eggs laid by females, the total hatchability of those egg laid, and the propensity of females to remate three and seven days after initial mating. For egg laying and hatchability, we used a linear regression model with latitude and longitudinal coordinates as predictors as well as the male and female identity and phenotyping blocks to account for the variation from genotypes of male and females in addition to experimental block effects. Model fit and effects of factors was assessed with ANOVA tables produced by the models. Because remating was scored as a categorical variable of whether or not the female copulated on the two remating days, we used logistic regression models to assess the effects of geographic location while controlling for male and female genotypes and block effects on short- and long-term female receptivity to remating. The significance of longitudinal and latitudinal coordinates and model fits were assessed using analysis of deviance tables.

We performed a permutation test to investigate the significance of the lower hatchability rates in the three central locations as revealed by linear models as well as visual confirmation of plots. We calculated the difference in hatchability between the five lines from our three central locations and the hatchability of all other fly lines (18 lines). We then randomly assigned fly lines into groups of five and 18 and calculated the difference in hatchability between these two groups. These permutations were repeated 10,000 times. P-values were calculated by the number of times the difference in hatchability between these two groups were equal to or greater than our observed value divided by our 10,000 permutations. The line with the lowest hatchability was removed for a follow-up permutation test to confirm that the lower hatchability was only due to the effect of one line. Similar permutation tests was conducted on total egg counts to

determine that lower hatchability was also not due to lower egg counts. Hatchability of eggs laid by females mated to representative 'American' and 'Caribbean' males were performed separately, and P-values from these tests were corrected using the Bonferroni method.

All analysis was performed in R and the code for the permutation test is available upon request.

Longevity data analysis

Survival analysis is used for temporal data of waiting times to an event with censored data. We employed methods from survival analysis to examine our data. We analyzed the waiting times of female death after homotypic or heterotypic mating. Females that escaped or survived past our observational periods were considered censored data points. The first step of survival analysis is to estimate survival functions for each of our crosses, S(t), which in our study is the probability of a female living longer than time, t. This can be done non-parametrically using the Kaplan-Meier method (Kleinbaum and Klein 2012). Parametric models were tested (i.e. exponential, log-normal, log-logistic, and generalized gamma), but none yielded a good fit (data not shown). After survival curves were fitted, we used it to estimate the cumulative hazard function, H(t), for each type of cross. The cumulative hazard function shows the cumulative probability that a female has expired up to time, t. The relationship between the survival function and the cumulative hazard function is:

$$H(t) = -ln(S(t))$$

or

$$S(t) = e - H(t)$$

The most common statistical test used for comparing survival distributions is the log-rank test. However, this test has the proportional hazards assumption which requires that the hazard functions of the two groups being compared are parallel. Hazard functions for our comparisons of female longevity after

241 heterotypic and homotypic matings were plotted and visually checked for the crossing of hazard curves.
242 When hazard curves cross, the proportional hazards assumption is violated so another test must be
243 conducted because the standard log-rank test has little to no power (Klein and Moeschberger 1997). We
244 chose to use a combined weighted log-rank test, which takes into account crossing hazard curves (Zhou
245 et al., 2006). This improved log-rank test has more power than the standard log-rank tests when the
246 hazard functions cross and the hazard ratio is not proportional.
247
248 All analysis was performed in R using the 'survival' package to estimate the survival curves and hazard
249 functions. The package 'emplik' was used as part of the improved log-rank test where the R code can be
250 obtained online (http://www.ms.uky.edu/%7Emai/research/LogRank2006.pdf).
251
252 **Results**
253 *Egg counts*
254 Egg counts for each line are shown using side-by-side box plots with locations arranged from the
255 northernmost to the southernmost location, left to right (FIGURE 2A, 2B). It does not appear that egg
256 counts follow a clinal pattern in either case of females mated to representative 'American' or 'Caribbean'
257 males. There is much variation within lines, but the median egg count for each location is approximately
258 the same except for in the case of females from location 6 (line 6-1; Sebastian, FL) when mated to
259 Caribbean males (FIGURE 2B).
260
261 The full regression model showed that longitude and latitude were not significant influences ($p = 0.3324$)
262 on egg laying and that most variance was accounted for by male ($p < 0.001$) and female ($p<0.001$)
263 genotypes as well as block effects ($p = 0.0018$). Comparing the full model with the reduced model in
264 which longitude and latitude were omitted showed that the addition of longitude and latitude as predictive
265 variable did not help the predictive power of the full model ($p = 0.4994$). (SUPPLEMENTARY TABLE 2, 3,
266 4)
267
268 *Remating*

Short- and long-term remating rates for each isofemale line were plotted against latitude and longitude coordinates (SUPPLEMENTARY FIGURE 1, 2). Short-term remating rates were generally lower (range of rates : 0-30%) than long-term remating rates (range of rates: 0-60%). Remating rates do not appear to be influenced by location, which was investigated further with logistic regression.

The full logistic regression model evaluating effects of latitude and longitude while controlling for male and female genotypes as well as block effects found that latitude ($p = 0.11$) or longitude ($p = 0.35$) were not useful in predicting short-term remating rates with similar results for long-term remating rates (lon $p = 0.7616$, lat $p = 0.6361$). Male genotype also was not a significant influence on short-term or long-term remating rates ($p = 0.4848$ and $p = 0.1240$). The reduced models removing latitude and longitude as predictors also showed that they were not significantly influencing remating rates. Female identities in both logistic models for short- and long- term remating rates were significant giving evidence that female genotypes could influence remating rates. However, when we fitted a model for long-term remating rates with a male x female interaction term, results showed that this interaction term was not significant ($p = 0.0959$). (SUPPLEMENTAL TABLE 5, 6, 7, 8, 9, 10, 11, 12)

Hatchability

Hatchability for the various locations in the southeast US and Caribbean Islands were visualized using side-by-side box plots with locations arranged from the northernmost to the southernmost location, left to right (FIGURE 2C, 2D). Hatchability in the three middle locations (location 4, 28, 33) at the border of the southeast US and Caribbean Islands appear lower than the locations on the edges in both the graphs displaying hatchability of females mated to American males (Figure 2C) and Caribbean males (Figure 2D).

Our full linear regression model took into account male and female identities on hatchability as well as experimental block effects while assessing influences of longitude ($p = 0.048$) and latitude. Longitude had a significant effect on hatchability ($p =0.0483$) while latitude did not ($p = 0.2396$). However when we compare the reduced model with latitude removed with the full model, we find that latitude did help significantly in explaining hatchability (0.0302). (TABLE 2, 3, 4)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Block	14	2.9337	0.20955	4.6414	4.007e-08
Female	22	7.7220	0.35100	7.7745	<2.2e-16
Male	1	0.8869	0.88692	19.6448	1.084e-05
Latitude	1	0.6255	0.06255	1.3856	0.23956
Longitude	1	0.17674	0.17674	3.9147	0.04826
Residuals	694	31.3326	0.04515		

TABLE 2: ANOVA Table for Full Model of Hatchability

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Block	14	2.9337	0.20955	4.6168	4.557e-08
Female	22	7.7220	0.35100	7.7332	<2.2e-16
Male	1	0.8869	0.88692	19.5403	1.143e-05
Longitude	1	0.0264	0.02639	0.5813	0.446
Residuals	6945	31.5455	0.04539		

TABLE 3: ANOVA Table for Reduced Model of Hatchability with no Latitude

	Res Df	RSS	DF	Sum of Sq	F	Pr(>F)
Full	694	31.333				

Reduced	695	31.546	-1	-0.21291	4.47158	0.03022

TABLE 4: ANOVA table of model comparisons

To evaluate the significance of the dip in hatchability rates, we performed permutation tests as described in our methods section. We found that the hatchability in the middle three locations was significantly lower than the rates in the surrounding locations regardless of being mated to an American male ($p < 0.0001$) or Caribbean male ($p < 0.0001$). Results were similar when the location with the lowest hatchability rate was removed (28: Sebastian, FL, USA) and the permutation tests performed again (females mated to American male: $p = 0.0056$, females mated to Caribbean male: $p = 0.0272$). Similar tests were conducted on egg counts to investigate whether the lower hatchability was due to lower egg counts. No significant differences in egg counts between females from the middle locations and the outer locations were found regardless of whether they were mated to American males ($p = 0.3192$) or Caribbean males ($p = 0.7584$). The same results were yielded when we removed the influence of the middle location, 28: Sebastian, FL, USA, (mated to American males: $p=0.3016$, mated to Caribbean males: $p = 1.0$). These results suggest a generalizable central location effect on hatchability.

Longevity

Five female lines representing various locations in the Southeastern U.S. and Caribbean Islands were measured for longevity after experiencing homotypic or heterotypic matings. The homotypic cross survival curves for females from lines 3-1, 2-2, and 12-2 were consistently higher than the survival curves of females in heterotypic crosses (FIGURE 3, 4, 5). There were no apparent differences between homotypic and heterotypic survival curves of females originating from lines 7-2 or 8-1 (Figure 6, 7).

Hazard curves for all crosses and lines revealed non-proportional hazards in almost all cases of homotypic and heterotypic matings. (SUPPLEMENTARY FIGURE 3, 4, 5, 6, 7). Crossing points of all hazard functions were visually estimated for use in the improved log-rank tests (TABLE 5). The improved log-rank tests showed evidence that females after heterotypic matings had shorter lifespans than females in homotypic matings for females from lines 3-1 and 12-2 ($p = 0.0410$ and $p = 0.0271$). Females of line 2-

2 showed a reduced lifespan when involved in heterotypic matings (FIGURE 3), but these results were not significant (p = 0.3130).

Female line	T~approx time of crossing hazards	pvalue from improved log rank
13,34	37	0.04096407
40,23	42	0.4246727
33,11	40	0.6260448
H,25	23	0.02706502
20,17	61	0.3129819

TABLE 2.5: Improved Log-rank Test Results

Discussion

Speciation is a complex process, dependent on a plethora of factors including diverse selectional forces and interactions with the environment. We examined several potential postmating reproductive barriers including remating rates, egg laying rates, hatchability, and female longevity that may potentially influence a system in the early stages of sexual isolation.

We observed an interesting hatchability rate ‘valley’ produced by the isofemale lines originating from our three central locations spanning the border of the United States and the Caribbean Island. This result may be evidence that there are essential genetic differences between American and Caribbean fly populations, which could have manifested as an intrinsic postzygotic barrier between these two populations. This type of evidence is indicative of the presence of Bateson-Dobzhansky-Muller incompatibilities (DMI) which are negative epistatic interactions and the most common form of intrinsic postzygotic isolation (Presgraves, 2010). A reduction in the fitness of ‘hybrid’ offspring here restricts the product of gene flow between American and Caribbean *D. melanogaster* populations. A more thorough investigation of these lines and genome sequences that are beyond the scope of this study are required to confirm the presence of DMIs.

We examined female longevity postmating with males that were more or less genetically related to them as defined by physical distance. These results from the longevity assay were the inverse of our hatchability assays. Females originating from the central locations (i.e. location 7 and 8) did not seem as affected by heterotypic matings compared to females from the northern and southernmost locations (i.e. locations 2, 3, 12). Previous laboratory evolution studies indicate that females develop ‘resistance’ against males they coevolve with in the same environment (Arbuthnott et al. 2014). Our findings may support this theory in natural populations, however, due to the low number of lines we tested, our study is possibly lacking the power to appropriately detect the effects of this particular extrinsic postmating barrier.

We did not find any evidence that egg laying rates or remating rates influenced the reproductive success in a systematic way with regard to these isofemale lines from the southeast United States and Caribbean Islands. However, the lack of evidence from our study does not imply that behaviors are not influential postmating reproductive barriers. Current views of speciation view the process as a sliding continuum where speciation can move forward or step back and may even be arrested at intermediate stages (Seehausen et al. 2014). Depending on the driving force of speciation, different types of reproductive barriers form at particular stages (Seehausen et al. 2014) thus it may be that postmating behaviors could be of importance at other stages in the speciation continuum in which case other species in the *Drosophila* genus may be better candidates to further investigate this question.

While our findings contribute to the ever growing breadth of knowledge about sexual isolation and speciation, it also sheds light on the complexity of the interplay between different isolating mechanisms and genetic admixture. Overall our data suggests that long-term postmating consequences in terms of offspring fitness and female lifespan reduction are of greater influence in this particular incipient sexual isolation scenario when compared to short-term postmating behavioral responses such as egg laying and remating receptivity. We have also identified the border between the United States and Caribbean islands as a potential region where *D. melanogaster* populations are particularly admixed leading to interesting interactions between partial isolating mechanisms. Greater genetic admixture in flies originating from this

area could promote the lower hatchability of eggs laid by females from these populations if American and Caribbean flies are genetically distinct enough to increase the possibility of DMIs occurring. The same genetic admixture could also be contributing towards female hardiness against harm from mating with a wider range of genetically diverse males, which in turn can compensate for lower hatchability by increasing reproductive lifespan. Genome resequencing efforts of *D. melanogaster* individuals from this study system will help determine the amount of genetic mixing occurring in the southeast US and Caribbean islands.

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485 ***Figure Legends***

486 FIGURE 1: Map of locations used in postmating assays

487

488 FIGURE 2: Egg counts of females mated with A) American males and B) Caribbean males. Hatchability
489 of females mated with C) American males and D) Caribbean males. Each box plot is a isofemale line
490 arranged from the northernmost location (left) to the southernmost location (right)
491
492 FIGURE 3: Survival curves of females from line 2-2 after experiencing homotypic (solid line) or
493 heterotypic (dashed line) matings
494
495 FIGURE 4: Survival curves of females from line 12-2 after experiencing homotypic (solid line) or
496 heterotypic (dashed line) matings
497
498 FIGURE 5: Survival curves of females from line 3-1 after experiencing homotypic (solid line) or
499 heterotypic (dashed line) matings
500
501 FIGURE 6: Survival curves of females from line 7-2 after experiencing homotypic (solid line) or
502 heterotypic (dashed line) matings
503
504 FIGURE 7: Survival curves of females from line 8-1 after experiencing homotypic (solid line) or
505 heterotypic (dashed line) matings
506

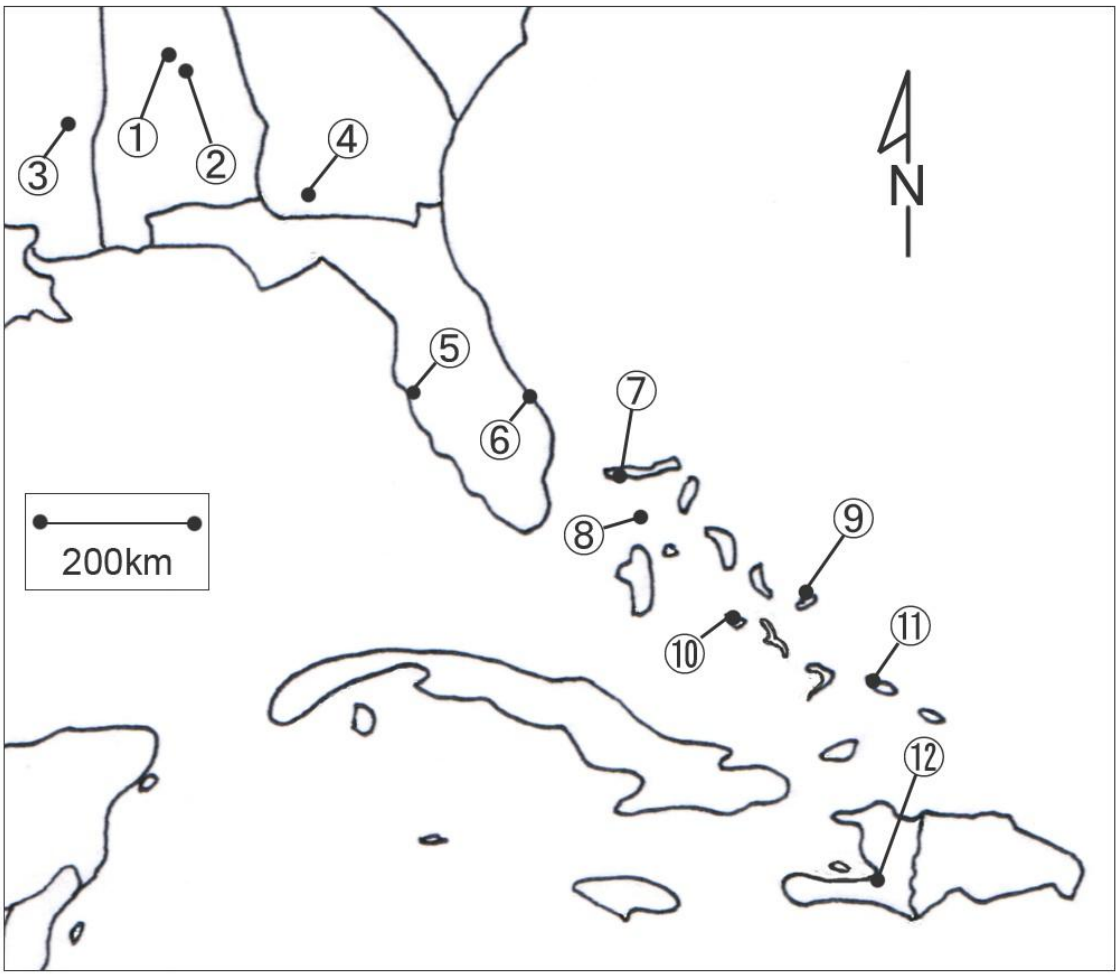


FIGURE1: Map of locations used in postmating assays

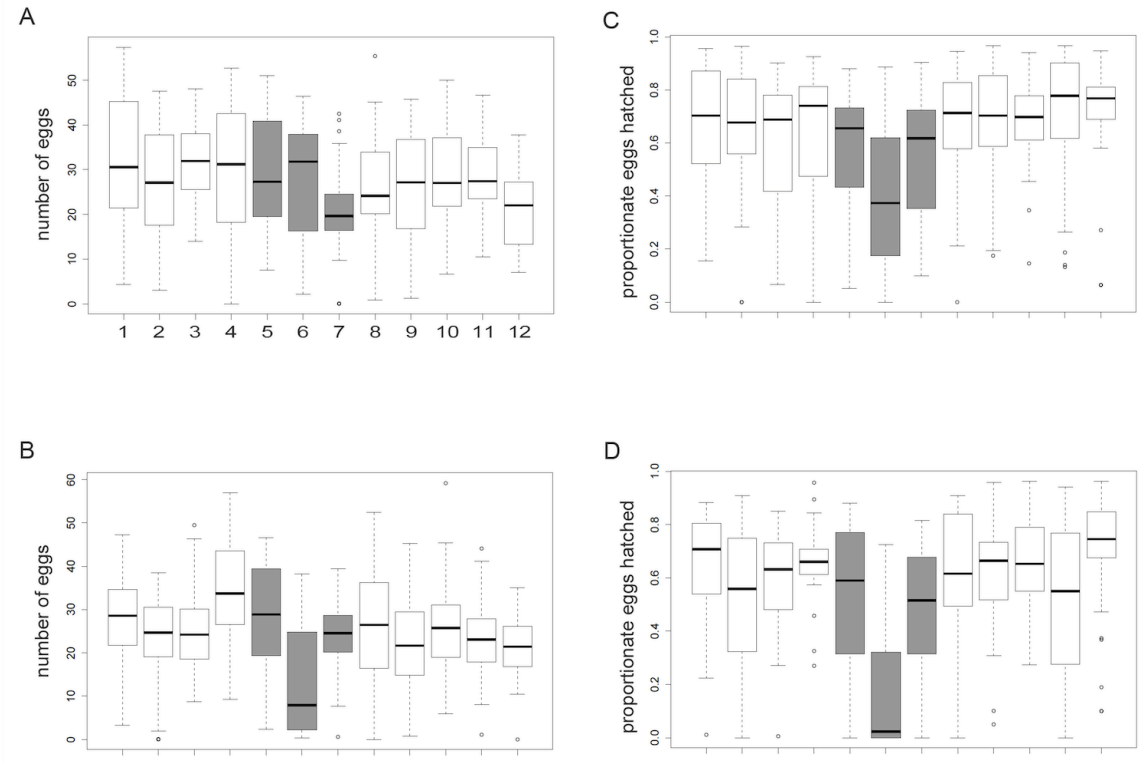
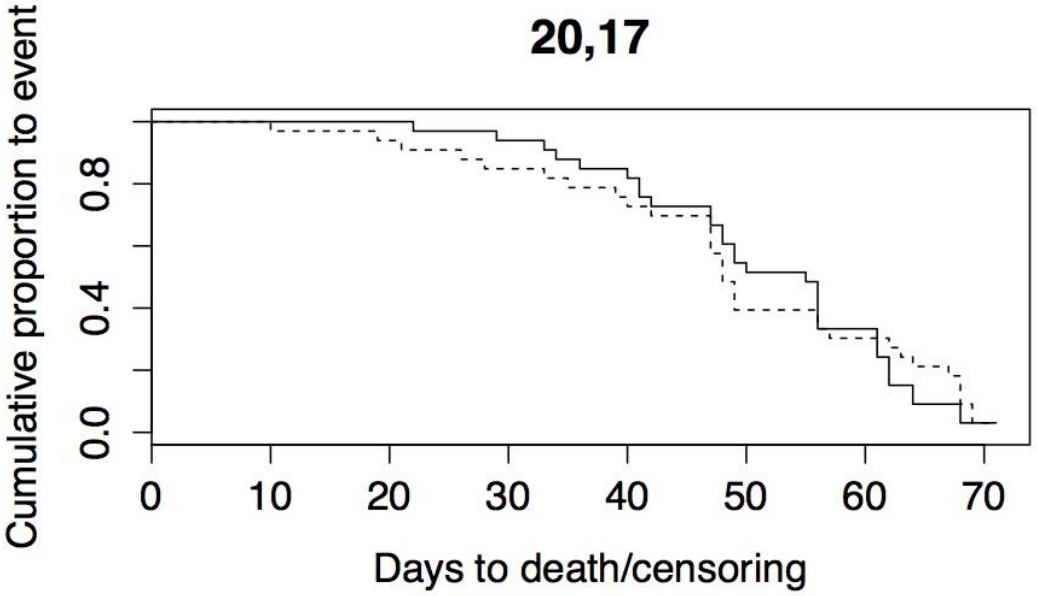


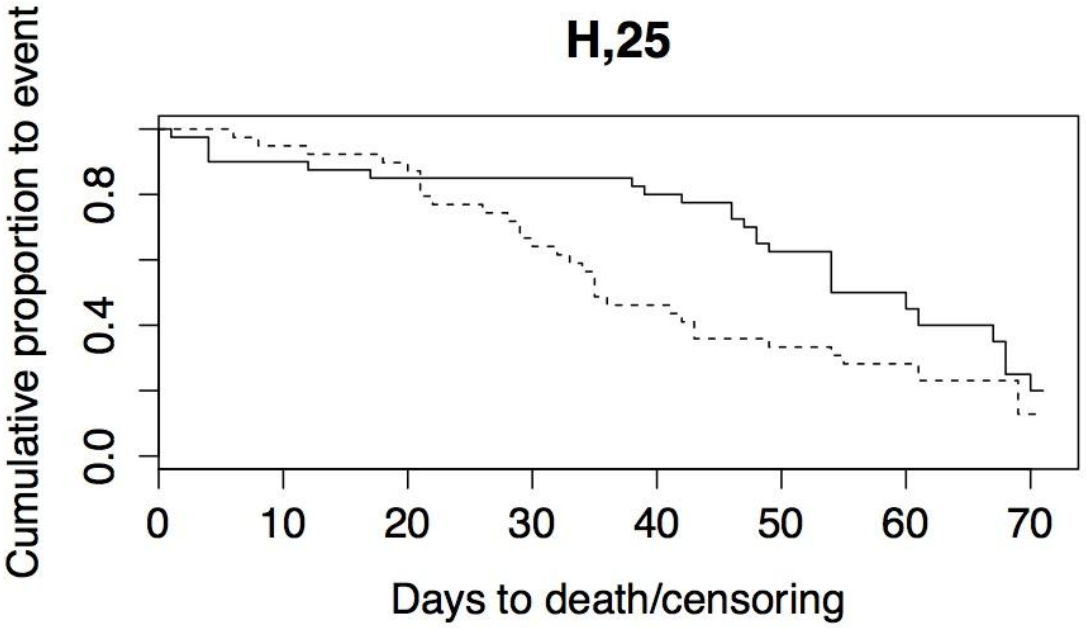
FIGURE 2: Egg counts of females mated with A) American males and B) Caribbean males. Hatchability of females mated with C) American males and D) Caribbean males. Each box plot is a isofemale line arranged from the northernmost location (left) to the southernmost location (right)



514

515 FIGURE 3: Survival curves of females from line 20,17 after experiencing homotypic (solid line) or
516 heterotypic (dashed line) matings

517



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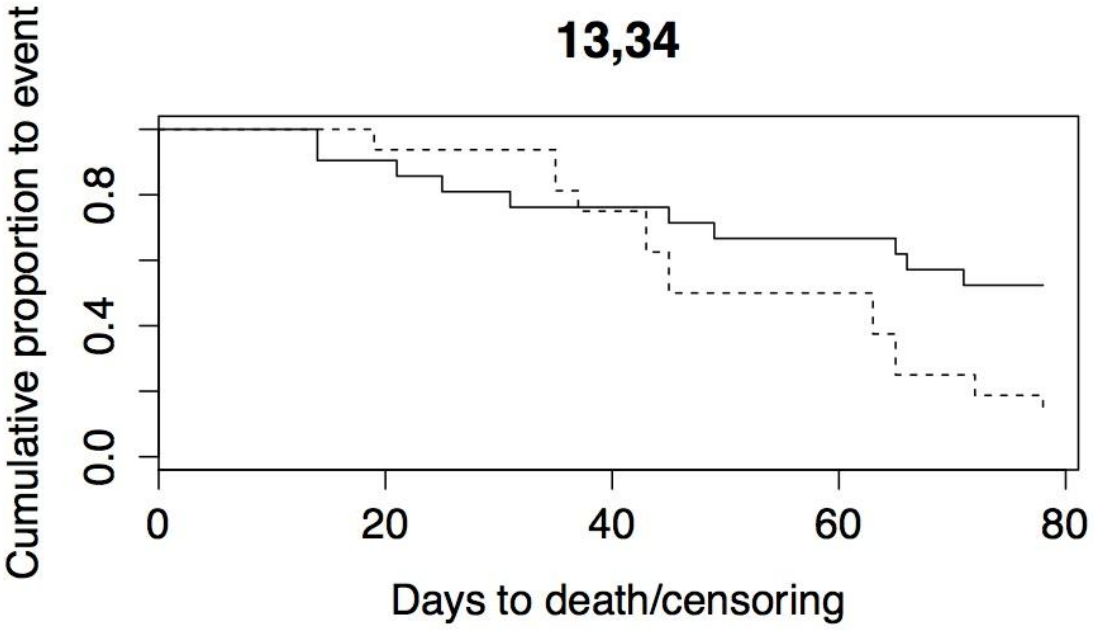
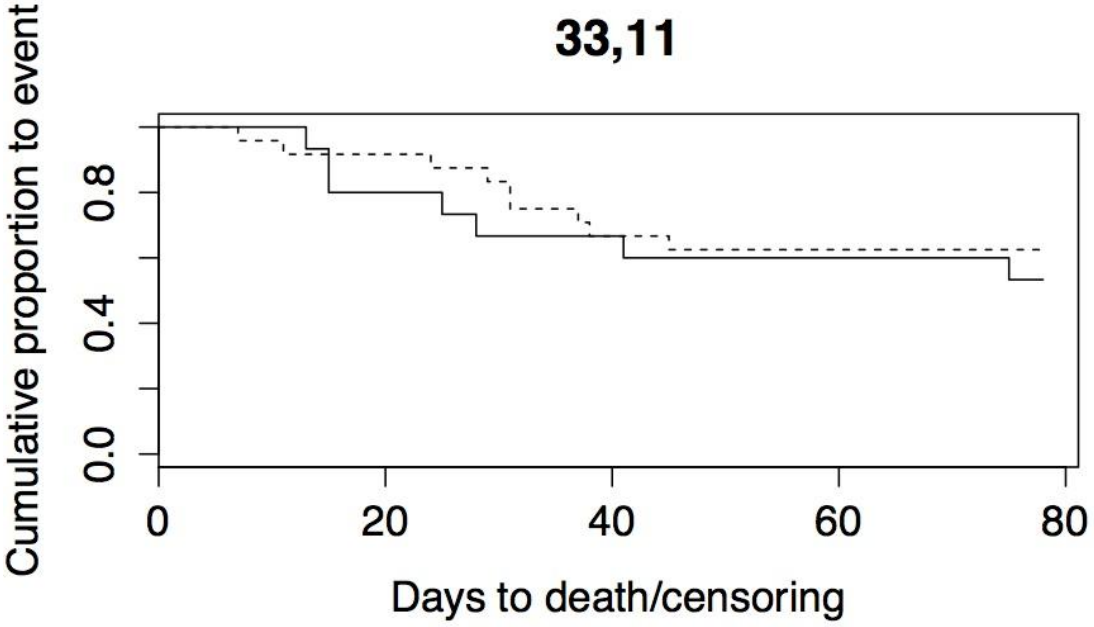
519 FIGURE 4: Survival curves of females from line H,25 after experiencing homotypic (solid line) or
520 heterotypic (dashed line) matings

521



522
523 FIGURE 5: Survival curves of females from line 13,34 after experiencing homotypic (solid line) or
524 heterotypic (dashed line) matings

525



526
527 FIGURE 6: Survival curves of females from line 33,11 after experiencing homotypic (solid line) or
528 heterotypic (dashed line) matings

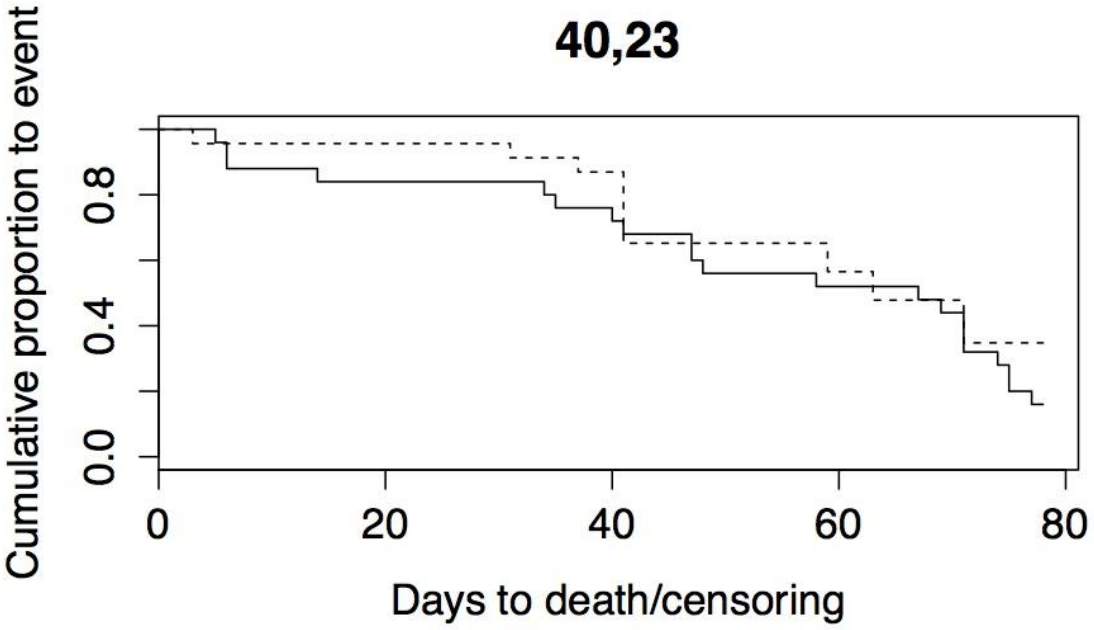


FIGURE 7: Survival curves of females from line 40,23 after experiencing homotypic (solid line) or heterotypic (dashed line) matings