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Reproductive Isolation through Experimental Manipulation of Sexually Antagonistic Coevolution in *Drosophila Melanogaster*

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Abstract

Promiscuity can drive the evolution of sexual conflict before and after mating occurs. Post-mating, the male ejaculate can selfishly manipulate female physiology leading to a chemical arms race between the sexes. Theory suggests that drift and sexually antagonistic coevolution can cause allopatric populations to evolve different chemical interactions between the sexes, thereby leading to post mating reproductive barriers and speciation. There is, however, little empirical evidence supporting this form of speciation. We tested this theory by creating an experimental evolutionary model of *Drosophila melanogaster* populations undergoing different levels of interlocus sexual conflict. We found that sexual conflict can cause reproductive isolation in allopatric populations through the co-evolution of chemical (post mating prezygotic) as well as behavioural (pre-mating) interaction between the sexes. Thus, to our knowledge, we provide the first comprehensive evidence of post mating (as well as pre mating) reproductive barriers due to sexual conflict.

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Introduction:

In most promiscuous species, males and females have differential reproductive investment and, consequentially differential evolutionary interest in the outcome of sexual interactions [1, 2]. This often leads to a scenario where adaptions benefitting one sex come at the expense of the other [3-5], ensuing a co-evolutionary chase typically called sexually antagonistic coevolution (SAC) [6]. According to verbal [7, 8] and formal [9, 10] arguments, SAC can lead to perpetual arms race between males and females of the same species. A byproduct this is the continual divergence between allopatric populations in genes related to reproduction, leading to reproductive isolation (RI) even in the absence of natural selection. This hypothesis is supported indirectly, by comparative studies that showed higher rates of speciation in insect clades with sexual conflict than those without [11]. However, no such evidence is found in other studies on mammals, butterflies, spiders [12] and birds [13].

An alternative to phylogenetic analysis that has been used to directly test the hypothesis is through experimental evolution which generally follows a simple experimental design:

a. Evolving independent replicate (i.e., allopatric) populations maintained under high and low/no conflict regimes (e.g., by enforcing monogamy or altering sex ratio) while all else remains equal.
b. Thereafter quantifying RI between allopatric populations within a regime and comparing the extent of isolation between different regimes.

As per the hypothesis, then, upon secondary contact allopatric populations will show evidence of RI that is relatively stronger, if not detectable only within the high conflict regime. Martin and Hosken tested the hypothesis in *Sepsis cynipsea* by evolving replicate populations under polygamy (SAC) and monogamy (removal of SAC) for 35 generations. They found that allopatric pairs showed significantly less mating success compared to their sympatric counterparts in the polygamous, but not in monogamous regime, thus providing the

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first evidence that antagonistically evolving behavioral traits can lead to reproductive isolation [14].

Along with pre-mating behavioral interactions, post-mating chemical interactions are important players in driving SAC. Ejaculate-female interaction and subsequent coevolution has been shown to have caused diversification in both ejaculate components (e.g. sperm, accessory gland proteins, small molecules transferred through ejaculate) and female reproductive tract and behavior across taxa [15]. Thus, post-mating antagonistic coevolution can lead to post-mating RI through an 'assortative sperm/ejaculate choice' process that is analogous to assortative mate choice. However, there is no empirical evidence favouring this. Despite multiple studies testing the hypothesis in different organisms, the study by Martin and Hosken remains the only direct evidence of SAC as a driver of RI so far [16-20], and the idea of sexual conflict as an 'engine of speciation' remains controversial [21].

We used two sets of allopatric populations of *Drosophila melanogaster* – one set (three populations) evolving under male biased (M) operational sex ratio and the other set (of three populations) evolving under female biased (F) operational sex ratio, demonstrating high and low levels of SAC respectively [4,5]. We tested whether reproductive isolation between allopatric populations was more prominent, if not present only in M as compared to F regime.

Reproductive isolation can manifest in three stages: premating, postmating prezygotic and postzygotic isolating barriers [22]. We have focused on the first two as they are expected to evolve rapidly and have greater chance of being manifested [7] within the relatively shorter time scale of experimental evolution.

As a measure of premating isolation, we assayed (a) assortative mating between females and males from the same population in presence of a competitor male from a different population (within the same regime) and (b) female reluctance to mate. As for postmating prezygotic

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isolation, we compared (a) copulation duration and (b) competitive fertilization success of males from within and across population crosses.

We found evidences of both premating and postmating prezygotic RI between populations in M regime but not in F, prompting us to conclude that sexual conflict can indeed act as 'an engine of speciation'.

Results:

The selection lines were derived from a long term laboratory adapted population of *Drosophila melanogaster* called LH_{st}[24]. The LH_{st} population, in turn was derived by the introgression of an autosomal 'scarlet eye' (st) mutation to another large laboratory bred population called LH (see methods for further description of ancestral populations).

Each of the three independent replicates of male biased regime ($M_{1,2,3}$) and female biased regime($F_{1,2,3}$) were created by altering the sex ratio to 1:3::female: male and 3:1::female: male respectively(23). All assays were done between the 95^{th} and 105^{th} generations of selection.

Males and females used in the assays were either from the same replicate population or from different replicate populations within a regime, which we term as 'within replicate' (WR) and 'between replicate' (BR) respectively. Flies used for all the assays were collected as virgins and a held singly in vials (90-mm length × 30-mm diameter) containing fresh corn meal - yeast- molasses food. All flies were 2-3 day-old adults at the time of assay.

Assay for premating isolation:

To look for pre-mating reproductive isolation though assortative mating, we combined a virgin female with a WR and a BR virgin male (simultaneously) in a round-robin manner and observed which one of the two males mated with the female(Table 1). A binomial test for each of the combinations displayed evidence of positive assortative mating (i.e., proportion of

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101 successfully mated WR males was significantly higher than random mating expectancy of
102 0.5) for all three replicates of the M regime, while none of the F regime showed the same
103 (Table 2). A t-test comparing selection regimes for the three replicates also shows that WR
104 males have significantly higher competitive mating success in M than in F (p= 0.02, Table 2).
105 This suggests pre-mating reproductive isolation between allopatric in the M regime.
106 However, another measure of pre-mating isolation, mating latency (time taken for a pair to
107 start mating after they are combined) showed no evidence of reproductive isolation (two way
108 ANOVA: $F_{1,226}$=1.679, p = 0.614; One way ANOVAs: $F_{1,116}$= 0.0104, p=0.9188 and $F_{1,109}$ =
109 0.4874, p = 0.4866 respectively; Fig S1, S2, S3).

110 **Table 1: Mating treatments for different assays. The letters i and j denote block**
111 **(reolicate) numbers, i ≠ j (in a round robin way). All mating trials were conducted**
112 **within a selection regime.**

Assay	Female from block	Male from block	Sample size
Assortative mating	**i**	**i(pink) + j(green)** **j(green) +i(pink)**	**30** **30**
Mating latency, copulation duration, Sperm defence ability	**i**	**i**	**20**
	i	**j**	**20**

113

114 **Table 2: Results of the assay for assortative mating show that in M regime, assortative**
115 **mating happens favouring WR males and females- compared to random expectation**
116 **(binomial test) and F regime (t test).**

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'k (success)' denotes number of successful mating between WR males and females out of n trials performed in each category. The 'binom prob' shows the probability of finding k successes out of n trials if matings were random (success probability = 0.5). We considered a probability of <0.05 to be significant. The't test results' column depicts t test results comparing the proportion of successful WR males in F and M regime. Proportion of successful WR male was arcsine square-root transformed to meet the assumptions of parametric analysis.

SelReg	Block	k(success)	n(trials)	binom prob (p=0.5)	Arcsinesqrt (prop.success)	t test results
F	1	26	56	0.092238	0.749653	t Ratio = 3.79607
F	2	18	56	0.002947	0.636217	DF = 4
F	3	28	58	0.10084	0.768153	Prob>|t|=0.02
M	1	33	56	0.043955	0.87139	Prob> t=0.01
M	2	33	54	0.028885	0.949017	
M	3	34	58	0.044521	0.872038	

Assay for post-mating prezygotic isolation:

To test for post-mating prezygotic isolation, we first measured copulation duration (the time spent *in-copula* by a mating pair). Within each selection regime we had two treatments where one virgin female was combined with either one virgin BR or one virgin WR male. We had

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129 60 replicate vials per treatment (WR/BR) per selection regime (M/F) for this experiment
130 (table1).

131 In a two way ANOVA using treatment and selection as fixed factors, we found a significant
132 selection regime × treatment interaction ($F_{1, 226}$= 4.269, p = 0.04, Fig 2). Both Tukey's HSD
133 and one way ANOVAs performed separately on the two selection regimes using treatment as
134 a fixed factor showed that in F, there was no difference in copulation duration ($F_{1,116}$ = 0.022,
135 p=0.883)(Fig S3) but in M, copulation duration was significantly higher in WR crosses
136 compared to BR crosses ($F_{1,109}$ = 8.834,p=0.003)(Fig S4).

137 **Fig1. Figure1: Mean copulation duration (±S.E) of WR and BR treatments from female**
138 **biased (F) regime male biased (M) regimes based on the results of two-way ANOVA.**
139 Points not sharing common letter (e.g., A and B) are significantly different based on Tukey's
140 HSD.
141

142 The difference in copulation duration was an indication of incipient reproductive isolation in
143 terms of reproductive behavior. We have previous evidence that in the ancestral population,
144 copulation duration of the first mating is positively correlated with sperm defense ability [25].
145 So we tested if such behavioral change translates into fitness difference. Sperm defense
146 ability (P1) is measured as the proportion of progeny sired by the first male when the female
147 is mated with multiple males (typically two males for assay purposes). A two way ANOVA
148 similar to that of mating latency and copulation duration showed a significant selection
149 regime × treatment interaction ($F_{1, 311}$=3.981, p = 0.046, Fig 3). Both Tukey's HSD and one
150 way ANOVAs performed separately on the two selection regimes using treatment as a fixed
151 factor showed that in F, P1 of WR and BR males were not different ($F_{1,170}$ = 0.0199, p=
152 0.8879) (Fig S5) but in M, WR males had significantly higher P1 value compared to that of
153 the males from BR crosses ($F_{1,143}$ = 9.0121, p=0.0032)(Fig S6). This indicates that the
154 difference in mating behavior also translates into fitness differences.

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Fig2. Mean (arcsine square root transformed) p1 (±S.E) of WR and BR treatments from female biased (F) regime male biased (M) regimes based on the results of two-way ANOVA. Points not sharing common letter (e.g., A and B) are significantly different based on Tukey's HSD**.**

Discussion:

In this study, we used experimental evolution to show that high levels of SAC can lead to the evolution of early stages of reproductive isolation at (a) premating and (b) postmating prezygotic stages in populations of *Drosophila melanogaster*.

a. **Premating isolation**: We found that in populations under high sexual conflict (M), females mated primarily with males of the same population in presence of an allopatric competitor from the same regime. Populations under low sexual conflict (F), on the other hand, display no such trend. Our observations corroborate that of Martin and Hosken [14], who found evidences of premating isolation in dung fly populations maintained under promiscuous (but not in monogamous) conditions. However, unlike them, we did not find any difference in females' reluctance to mating (measured as mating latency in our study) under non-competitive scenario. This could be due to the fact that we used virgin females in our assay and there exist little variation in their reluctance to mate, as has been seen in previous studies testing the same hypothesis [14, 16-20]. Alternatively, in the M populations SAC might have created genetic divergence which manifests only under a choice scenario where females get spotted faster and/or courted more vigorously by WR males or simply find WR males more attractive than their BR counterparts. Thus, we provide evidence that premating RI can manifest itself under competitive scenario in terms of *mate choice* behavior in addition to/instead of failed mating or 'reluctance to mate' behavior – a possibility that has largely been neglected by most previous studies [16-19]. However, Plesnar-Bielak et al address this possibility but find no effect of SAC on assortative mating in the bulb mite *Rhizoglyphus*

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robini, after maintaining them under monogamous or polygamous regimes for 45 generations [20].

b. **Postmating Prezygotic RI:** Our assays resulted in WR pairs mating for longer and males enjoying greater sperm defense ability (when competed with common baseline males) than their BR counterparts in M populations but not in F. Thus in these populations SAC seems to have resulted in postmating prezygotic RI between allopatric populations.

Copulation duration is an important indicator of male ejaculate investment as well as cryptic male mate choice [30, 31]. In a similar study on *Drosophila pseudoobscura*, Bacigalupe et al used copulation duration as a one of the measures of reproductive isolation. In that, they evolved populations under different intensities of SAC and compared difference in copulation duration (among other traits) between WR and BR crosses. They found significant difference only in the regime with the highest SAC intensity, where WR crosses had lower copulation duration than BR crosses [18]. Our result is in stark contradiction to that. Copulation duration has also been used as an indicator of reproductive isolation in speciation studies on several *Drosophila* species complexes [26-28]. In all the studies, individuals from sister species did mate but, at least in some cases heterospecific matings had lower copulation duration than conspecific matings. Our results could represent an early stage of speciation in this regard. Lower copulation duration in BR mating compared to WR mating in M populations could be due to genetic divergence caused by SAC that leads to reduced ejaculate transfer ability and/or cryptic male investment by the males when they mate with allopatric females.

A number of studies - while testing if SAC drives reproductive isolation using experimental evolution - have measured post-mating isolation extensively in terms of difference in fecundity [17], offspring number [19, 20], offspring viability [17, 18] or offspring sterility [18], but found no evidence of isolation in those traits. An important area where RI can be manifest is competitive fertilization success [8] which none of the studies thus far has addressed. We find that M males have lower competitive fertilization success when

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competition happens in BR females than when it does in WR females, while in F males there is no such difference. Since in these populations it was not possible to assay direct sperm competition between BR and WR males, we have used a proxy measure where all the competitor males used in these assays were taken from the same ancestor population with the assumption that relative sperm competitive ability of the common competitors do not differ across replicate populations within a regime. This is a valid assumption since in a previous study comparing sperm competitive ability of M and F males (where we used the same common competitors) we found no replicate effect [23].

There are at least two reasons why M males have reduced sperm competitive ability when mated with allopatric M females. First, it could be a direct correlate of decreased copulation duration. Males with lower copulation duration do not/cannot transfer as much ejaculate and therefore have lower competitive ability [29]. The copulation duration-competitive ability correlation has been demonstrated in the ancestral population from which the selected populations have been derived [25]. Second, it could be a putative stage of conspecific sperm precedence (CSP) –where sperm of conspecific male has greater competitive success over that of heterospecific male. Evidence of CSP is widespread across various taxa [30-33] and its mechanisms have been illustrated for at least one set of Drosophila sibling species [32, 33]. In *Drosophila melanogaster* (as in most promiscuous species) females mate multiple times and often store ejaculate (in specialized storage organs, e.g., seminal receptacle and spermatheca in fruit flies) from different males where they compete for fertilization success. The outcome is mostly determined by how the resident ejaculate (from an earlier mating) is displaced from female storage organs by ejaculate from more recent mating [34] and is influenced by competing males and host female [35]. This provides ample scope for sperm-female coevolution [36]. Since at least some accessory gland proteins are harmful to females, ejaculate- female coevolution should be antagonistic in nature. Thus it is possible that increased postmating SAC drove divergence in replicate M populations in terms of how

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ejaculate and female reproductive tract interact to determine fertilization success leading to an incipient form of CSP. Thus, our results show higher rates of SAC can drive reproductive isolation in allopatric populations through reduced post-mating competitive success of males. Out of all the studies that have used experimental evolution to test the theoretical prediction that sexually antagonistic coevolution can drive reproductive isolation, there are only two (including the present one) that provide evidence in support, and to the best of our knowledge, this is the only one that provides evidence of post-mating isolation. There are multiple reasons as to why our results differ from most of its predecessors [16-20]:

a. The census population size for each replicate was bigger in our study than those of the previous ones.
b. The number of generations in those studies were too low (our assays were done after ~100 generations of selection compared to that of ≤ 50 in all of the previous studies) to allow SAC to drive population divergence to a degree where they are apparent.
c. According to theoretical predictions, reproductive isolation in allopatric populations is one of the six possible outcomes of sexual conflict [9]. It is possible that the populations under high SAC in those studies did not diverge with respect to each other. However, none of the studies shed ight upon any of the other five possibilities that might have occured in their populations.

In conclusion, we show direct evidence of evolution of both premating and postmating prezygotic RI as a consequence of SAC. Thus, it remains a distinct possibility that sexual conflict can result in a coevolutionary chase between the sexes [11, 37] and can indeed be 'an engine of speciation'. We speculate that initial genetic variation and number of generations can be important to realize – at least in experimental evolution studies –the evolution of RI caused by sexual conflict. However we also feel the need of more such

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256 studies to experimentally determine the exact conditions under which sexual conflict acts
257 as 'an engine of speciation' and to elucidate the underlying proximate mechanisms.

258 **Methods:**

259 **Ancestral Populations:**

260 LH – It is a large laboratory adapted population of *Drosophila melanogaster*, established by,
261 and named after Lawrence G Harshman. The population is maintained on a 14 day discrete
262 generation cycle, under 25^{o}C, 60-80% relative humidity, 12 hours light / 12 hours dark
263 (12hrs: 12hrs L/D cycle) and on standard cornmeal – molasses – yeast food. The flies are
264 grown under moderate larval density of 140-160 per 8-dram vial (25mm diameter × 90mm
265 height) containing 8-10ml food. On the 12th day post egg collection, flies from different vials
266 are mixed and redistributed across fresh food vials containing limiting amount of live yeast
267 grains with 16 males and 16 females per vial. On the 14th day, flies are transferred to fresh
268 vials and are allowed a window of 18 hours to lay eggs which (after discarding the adults and
269 controlling density) start the next generation (38).

270 LH_{st} –this population was derived by introducing the scarlet eye colour (recessive, autosomal
271 and benign) gene into the LH population, hence the subscript. LH_{st} is maintained under the
272 same condition as LH with N_e>2500. The genetic backgrounds of these two populations are
273 homogenized by periodic back crossing.

274 **Selection Regimes:**

275 The study was done on six populations of *Drosophila melanogaster* – $M_{1-3}$and F_{1-3}
276 representing male biased and female biased operational sex ratio respectively. All these
277 populations were created from the LH_{st} population.

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We derived the male biased (M_{1-3}) and female biased(M_{1-3}) regimes, each having three independent replicates, from LH_{st} by varying the operational sex ratio to male: female :: 3:1 and 1:3 respectively. The maintenance of these populations differs from that of LH/LH_{st} in the following ways:

(a) In these populations adult flies are collected as virgins 9-10 days after egg collection, during the peak eclosion period and held in vials (containing 8 flies of one sex) for two days.

(b) The sexes are combined on the 12^{th} day in fresh food vials seeded with measured amount of live yeast (0.47mg per female) following the selection regime – 24males+8females in each vial for M and 8males+24females in each vial for F.

The effective population sizes of all the populations are maintained at > 450 or >350 depending on the method used to calculate them (4). For more details on the evolutionary history and detailed maintenance protocol, see (23).

Standardization and Generation of Experimental Flies:

In order to equalize the potential non-genetic parental effects across different regimes , we maintained all populations under ancestral condition which does not include virgin collection and sex ratio alteration- essentially following the same life cycle as LH_{st} populations for one generation before obtaining individuals for the experiment. This process is called standardization (39).

Eggs laid by the standardized flies were collected to at a density of 150(±2) per vial (containing 8-10ml of cornmeal food) obtain the experimental flies. On the 10^{th} day after egg collection, males and females were collected as virgins during the peak of their eclosion and held as single individual per vial.

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Ancestral flies (LH), whenever they were used in this study, were raised in similar conditions. LH males were sorted on the 12th day post eclosion and held as single individuals. Eggs for LH flies were collected on the same day as that of the selection lines. Thus the age of the experimental flies of all the populations were same during the experiment.

General Experimental Design:

For all our assays, we compared reproductive behavior and/or fitness related traits between two types of individuals within a regime:

a. Within replicate (WR): These are individuals from the same replicate number of a given selection regime i.e., M_i♂ and M_i♀ are WR with respect to each other where i denotes the replicate number (e.g., M_1 ♂and ♀) and similarly for F.

b. Between replicate (BR): These are individuals from different replicate numbers of a given selection regime, i.e., M_i♂ and M_j♀ are BR with respect to each other –where i, j denote replicate numbers and (i,j) ∈ {(1,2), (2,3), (3,1)} (e.g. M_1♂ and M_2♀) and similarly for F. We took BR individuals in a round robin manner to avoid the problem of pseudo-replication (21).

Assay for Assortative Mating:

We combined a virgin female with two virgin males from the same selection regime –one WR and one BR – in vials containing fresh food. That is, a female from a given replicate number was combined with a male from the same replicate number and another from a different replicate number (all within the same selection regime), e.g., one M_1 female + one M_1 male + one M_2 male and so on. Thus we had three combinations within each selection regime, denoted by female replicate number. Males were marked by pink or green Day-Glo

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dust for identification. Previous studies using the same dust found no adverse effect on individuals (40). However, to account for any mating bias brought about solely by green and/or pink coloration, we had reverse coloration treatments for all combinations. Thus each combination had two treatments, e.g., one M_1 female + one green M_1 male + one pink M_2 male; one M_1 female + one pink M_1 male + one green M_2 male and so on. We had 30 replicate vials per combination per colour treatment (table1). In some vials we observed no mating till one hour after combining the flies. Those vials were discarded and excluded from analysis (the final sample sizes are listed in table 2 under the column 'n (trials)').

Assay for Mating Latency and Copulation Duration:

For this assay we combined one virgin male and one virgin female according to treatment (WR or BR, see results) in a vial containing fresh food. After combining a male and a female, the pair was observed till they finished mating. Time taken for a pair to start mating after they were combined was recorded as mating latency and the time they spent in-copula was recorded as copulation duration. If a pair failed to mate after one hour, they were discarded. However, the number of failed mating in all treatments was very low (6, 3, 0 and 3 failures out of 60 trials in M-WR, M-BR, F-WR and F-BR respectively). Mating latency and copulation duration values for each vial were used as the unit of replication.

Assay for Competitive fertilization success:

As a measure of competitive fertilization success, we measured sperm defense ability of males, the rationale for which is provided in the results section. For assaying sperm defense ability, we set up crosses following the same method as mentioned above and the vials were observed for mating for one hour. The females that did not mate with the first male were discarded. After the first mating, we sorted the females using light CO_2-anaesthesia and held them back into the vials and discarded the males. After allowing a recovery time (from

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anesthesia) of half an hour, we introduced a second male (red eyed, LH) in each vial and kept the vials undisturbed for 24 hours, during which they could mate with the females. After this exposure window, the second males were discarded and the females were transferred singly (under light anesthesia) to test tubes (dimensions: 12 mm diameter × 75 mm length) provisioned with food. There they were allowed an oviposition window of 18 hours. The adult progeny emerging from the eggs laid during this window were scored for their eye colour marker after 12 days. The proportion of scarlet progeny was taken as an estimate of P1 of the male. 90 males from each of the crosses were assayed for P1. Since we did not observe the second mating, instances where all progeny was sired only by the first male (P1=1) could arise due to second male failing to mate. Such instances were excluded from the analysis. Final sample size for P1 analysis was n=83-87 and 70-73 per cross type (WR/BR) in F and M populations respectively. P1 value from a single vial was used as the unit of replication.

Statistical Analysis:

To test for assortative mating, we used two different statistical analyses. First, we defined each mating as a “trial: and a female mating with WR male as “success” and used a binomial test with the assumption that both the males are equally likely to mate, i.e., p=0.5. If the probability of finding k successes out of n trials under this assumption was <0.05, we rejected the null hypothesis. We did this for each of the six combinations (2 selection regimes × 3 combinations each). Second, we calculated proportion of WR mating in each of the replicates, resulting in 3 values for M and F each and compared them using Student’s t-test. The proportion values were arcSine Square-root transformed to meet the assumptions of parametric test.

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For the rest of the assays, we performed a two-way ANOVA with selection regime and treatment (type of individuals involved in a cross: BR/WR) as fixed factors to test whether there was any interaction between the two factors in the measured observables. A significant interaction with greater isolation in M than in F would indicate presence of RI (*a la* 14, 16). We also performed one-way ANOVAs separately on the same data, but separately for each selection regime. The objective was to test whether WR and BR crosses differed in M populations or not. The F populations served as a control where it was expected that there would be no isolation (*a la 18*). We would like to point out here that the results remain quite robust and indicate to the same inference no matter which method is used to analyze the data.

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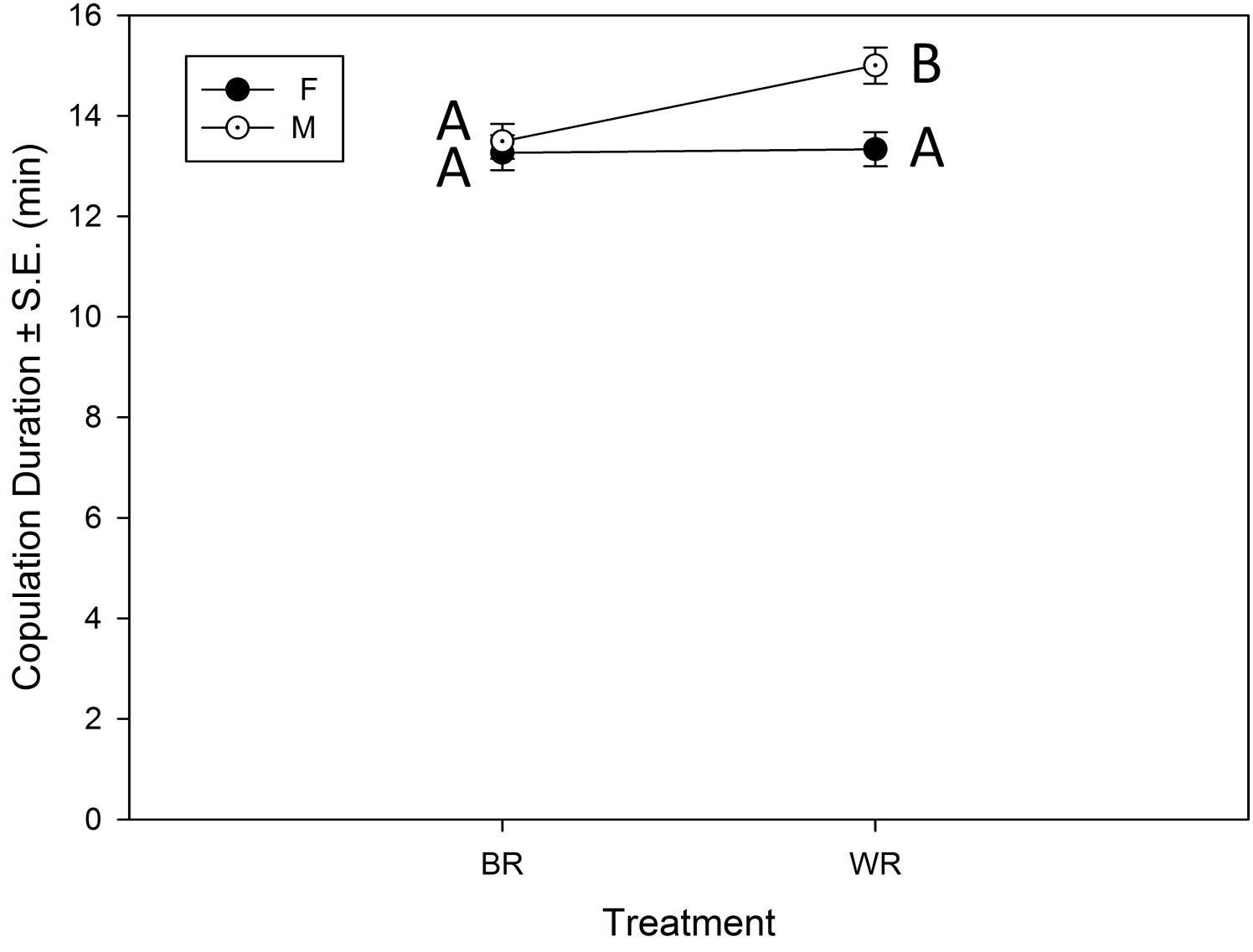
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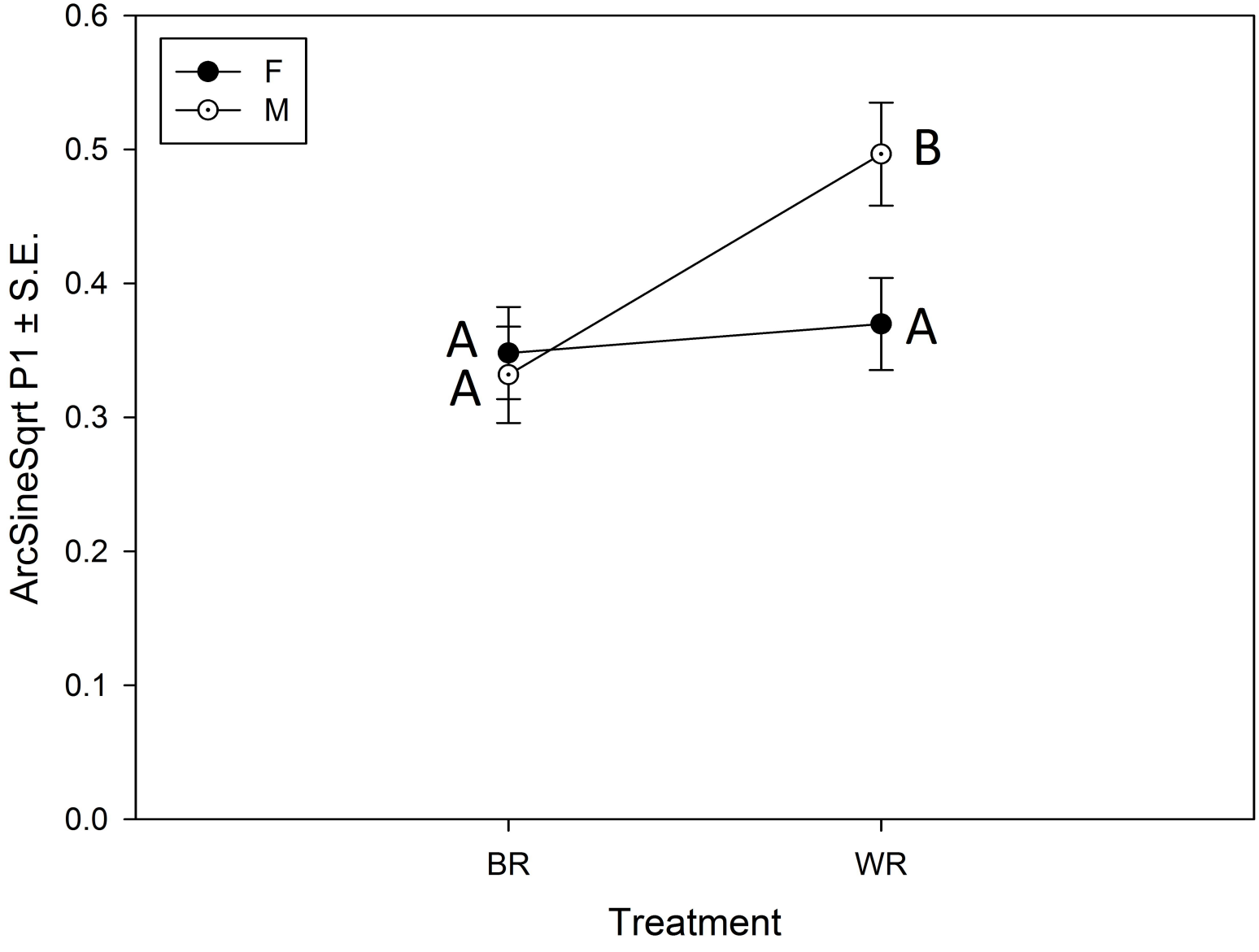
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