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1 **Reproductive Isolation through Experimental Manipulation of Sexually**
2 **Antagonistic Coevolution in *Drosophila melanogaster***

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13 ## Abstract

14 Promiscuity can drive the evolution of sexual conflict before and after mating occurs. Post-
15 mating, the male ejaculate can selfishly manipulate female physiology leading to a chemical
16 arms race between the sexes. Theory suggests that drift and sexually antagonistic coevolution
17 can cause allopatric populations to evolve different chemical interactions between the sexes,
18 thereby leading to postmating reproductive barriers and speciation. There is, however, little
19 empirical evidence supporting this form of speciation. We tested this theory by creating an
20 experimental evolutionary model of *Drosophila melanogaster* populations undergoing
21 different levels of interlocus sexual conflict. We found that allopatric populations under
22 elevated sexual conflict show assortative mating indicating premating reproductive isolation.
23 Further, these allopatric populations also show reduced copulation duration and sperm
24 defense ability when mating happens between individuals between individuals across
25 populations compared to that within the same population, indicating postmating prezygotic
26 isolation. Sexual conflict can cause reproductive isolation in allopatric populations through
27 the coevolution of chemical (postmating prezygotic) as well as behavioural (premating)
28 interaction between the sexes. Thus, to our knowledge, we provide the first comprehensive
29 evidence of postmating (as well as premating) reproductive isolation due to sexual conflict.

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30 **Introduction:**

31 In sexually reproducing species, males and females often have differential reproductive
32 investment, and, consequently differential evolutionary interest in the outcome of sexual
33 interactions[1, 2]. This often leads to a scenario where adaptations benefitting one sex come at
34 the expense of the other[3, 4, 5], ensuing a coevolutionary chase typically called sexually
35 antagonistic coevolution (SAC)[6]. According to verbal[7, 8] and formal[9, 10] arguments, SAC can
36 lead to a perpetual arms race between males and females of the same species. A byproduct of
37 this is the continual divergence between allopatric populations in genes related to
38 reproduction, leading to reproductive isolation (RI) even in the absence of natural selection.
39 This hypothesis is supported indirectly by comparative studies that showed higher rates of
40 speciation in insect clades where sexual conflict is observed than those where it is not
41 observed[11]. However, no such evidence is found in other studies on mammals, butterflies,
42 spiders[12] and birds[13].

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

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

50 Following this hypothesis, upon secondary contact, allopatric populations will show
51 relatively stronger evidence of RI in the high conflict regime compared to low/no conflict
52 regime. Martin and Hosken tested the hypothesis in *Sepsis cynipsea* by evolving replicate
53 populations under polygamy (SAC) and monogamy (removal of SAC) for 35 generations.
54 They found that allopatric pairs showed significantly less mating success compared to their

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55 sympatric counterparts in the polygamous, but not in monogamous regime, thus providing the
56 first evidence that antagonistically evolving behavioural traits can lead to reproductive
57 isolation[14].

58 Along with premating behavioural interactions, postmating chemical interactions are
59 important players in driving SAC. Ejaculate-female interaction and subsequent coevolution
60 has been shown to have caused diversification in both ejaculate components (e.g. sperm,
61 accessory gland proteins, small molecules transferred through ejaculate) and female
62 reproductive tract and behaviour across taxa[15]. Thus, postmating antagonistic coevolution can
63 lead to postmating RI through an 'assortative sperm/ejaculate choice' process that is
64 analogous to assortative mate choice. However, there is no empirical evidence favouring this.
65 Despite multiple studies testing the hypothesis in different organisms, the study by Martin
66 and Hosken remains the only direct evidence of SAC as a driver of RI so far[16,17,18,19,20], and
67 the idea of sexual conflict as an 'engine of speciation' remains controversial[21].

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

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

77 As a measure of premating isolation, we assayed (a) assortative mating between females and
78 males from the same population in presence of a competitor male from a different population

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79 (within the same regime) and (b) female reluctance to mate. As for postmating prezygotic
80 isolation, we compared (a) copulation duration and (b) competitive fertilisation success of
81 males from within and across population crosses.

82 Results:

83 The selection lines were derived from a long term laboratory adapted population of
84 *Drosophila melanogaster* called LH_{st}[23]. The LH_{st} population was in turn derived by the
85 introgression of an autosomal recessive 'scarlet eye' (st) mutation to another large laboratory
86 bred population called LH (see methods for further description of ancestral populations).

87 Each of the three independent replicates of female biased regime ($F_{1,2,3}$) and male biased
88 regime($M_{1,2,3}$) were created by altering the sex ratio to female biased (1 male:3 female) and
89 male biased (3 male: 1 female) respectively[24]. All assays were done between the 95th and
90 105th generations of selection.

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

96 Assay for premating isolation:

97 To look for premating reproductive isolation though assortative mating, we combined a
98 virgin female with a WR and a BR virgin male (simultaneously) in a round-robin manner and
99 observed which one of the two males mated with the female (Table 1). A logistic regression
100 showed higher degree of assortative mating in M regime compared to that in F, i.e., in M
101 regime, WR males mated more successfully compared to BR males (p= 0.0488). While the
102 log-odds ratio showed no difference in choice for F regime ("intercept" in Table 2), in M

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103 regime the ratio increases significantly for successful WR matings in M regime
104 ("SelectionM" in table 2) . This suggests premating reproductive isolation between allopatric
105 populations in the M regime. However, another measure of premating isolation, mating
106 latency (time taken for a pair to start mating after they are combined) showed no evidence of
107 reproductive isolation (two way ANOVA: $F_{1,221}$=1.678, p = 0.613, Figure 1).

108 **Table 1: Mating treatments for different assays. The letters i and j denote block**
109 **(replicate) numbers, i ≠ j (in a round robin way). All mating trials were conducted**

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	i	i	20
Sperm defence ability	i	j	30

110 **within a selection regime.**

111

112 **Table 2. Results of logistic regressionperformed on the data obtained from the assay for**
113 **assortative mating. Successful mating between WR individuals was used as the response**
114 **variable. Selection regime was used as fixed effect and replicate population nested**

	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-0.0470675	0.15343501	-0.3067586	0.759027
SelectionM	0.43273	0.21965409	1.9700521	0.048832

115 **within Selection regime was used as random effect.**

116

117 **Assay for postmating prezygotic isolation:**

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118 To test for postmating prezygotic isolation, we first measured copulation duration (the time
119 spent *in-copula* by a mating pair). Within each selection regime we had two treatments where
120 one virgin female was combined with either one virgin BR or one virgin WR male. We had
121 60 replicate vials per treatment (WR/BR) per selection regime (M/F) for this experiment
122 (Table 1).

123 In a two way ANOVA using treatment and selection as fixed factors, we found a significant
124 selection regime × treatment interaction ($F_{1, 221}$= 4.269, p = 0.03, Figure 2). Tukey's HSD
125 showed that in F there was no difference in copulation duration but in M, copulation duration
126 was significantly higher in WR crosses compared to BR crosses (Table 3).

127 **Table 3. A. Least square means ± S.E. values for Copulation Duration and P1.**

128 **B. P values obtained through pairwise contrast using Tukey's HSD. The important**
129 **contrasts have been highlighted in bold.**

A. Lsmeans			
Selection Regime	Mating Type	Lsmeans ±S.E.	
		Copulation Duration	ArcSineSqrt (P1)
F	BR	13.2359 ± 0.688966	0.349871± 0.0504
M	BR	13.47331 ±0.688967	0.329624±0.051427
F	WR	13.33333 ±0.68479	0.373822±0.050413
M	WR	15.01464± 0.695161	0.498448±0.053216

B. Contrasts (Tukey's HSD)		
Contrast	p.values	
	Copulation Duration	AecSineSqrt (P1)
F BR - M BR	0.959157	0.97628
F BR - F WR	**0.996832**	**0.959197**
F BR - M WR	0.001716	0.019729
M BR - F WR	0.990766	0.804703
M BR - M WR	**0.009053**	**0.007008**
F WR - M WR	0.003004	0.070632

130

131 The difference in copulation duration was an indication of incipient reproductive isolation in
132 terms of reproductive behaviour. We have previous evidence that in the ancestral population,
133 copulation duration of the first mating is positively correlated with sperm defense ability[25].

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134 So we tested if such a behavioural change translates into fitness difference. Sperm defense
135 ability (P1) is measured as the proportion of progeny sired by the first male when the female
136 is mated with multiple males (typically two males for assay purposes). A two way ANOVA
137 similar to that of mating latency and copulation duration showed a significant selection
138 regime × treatment interaction ($F_{1, 306}$=4.198, p = 0.041, Figure 3). Tukey's HSD and showed
139 that in F, P1 of WR and BR males were not different but in M, WR males had significantly
140 higher P1 value compared to that of the males from BR crosses (Table 3). This indicates that
141 the difference in mating behaviour also translates into fitness differences.

142

143 **Discussion:**

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

147 In populations under high sexual conflict (M), females mated primarily with males of the
148 same population in presence of an allopatric competitor from the same regime. Populations
149 under low sexual conflict (F), on the other hand, displayed no such trend. Our observations
150 corroborate that of Martin and Hosken[14], who found evidences of premating isolation in dung
151 fly populations maintained under promiscuous (but not monogamous) conditions. However,
152 unlike them, we did not find any difference in females' reluctance to mating (measured as
153 mating latency in our study) under non-competitive scenario. The most plausible reason for
154 this is the interspecies difference of the two studies. In Sepsis flies used in the previous
155 study[14], females show conspicuous reluctance by shaking and kicking. While such
156 conspicuous behaviour is not present in *Drosophila melanogaster*, we found that premating
157 isolation can manifest under a choice scenario. In the M populations SAC might have created

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158 genetic divergence, due to which females get spotted faster and/or courted more vigorously
159 by WR males or simply find WR males more attractive than their BR counterparts leading to
160 assortative mating. Thus, we provide evidence that premating RI can manifest itself under
161 competitive scenario in terms of *mate choice* behaviour in addition to/instead of failed mating
162 or 'reluctance to mate' behaviour – a possibility that has largely been neglected by most
163 previous studies[16, 17, 18, 19]. However, Plesnar-Bielak *et al*. address this possibility but find no
164 effect of SAC on assortative mating in the bulb mite *Rhizoglyphus robini*, after maintaining
165 them under monogamous or polygamous regimes for 45 generations[20].
166 Our assays resulted in WR pairs mating for longer and males enjoying greater sperm defense
167 ability (when competed with common baseline males) than their BR counterparts in M
168 populations but not in F. Thus in these populations, SAC seems to have resulted in
169 postmating prezygotic RI between allopatric populations.
170 Copulation duration is an important indicator of male ejaculate investment as well as cryptic
171 male mate choice[30, 31]. In a similar study on *Drosophila pseudoobscura*, Bacigalupe *et al.*
172 used copulation duration as a one of the measures of reproductive isolation. In that, they
173 evolved populations under different intensities of SAC and compared difference in copulation
174 duration (among other traits) between WR and BR crosses. They found significant difference
175 only in the regime with the highest SAC intensity, where WR crosses had lower copulation
176 duration than BR crosses[18]. Our result is in stark contradiction to that. Copulation duration
177 has also been used as an indicator of reproductive isolation in speciation studies on several
178 *Drosophila* species complexes[26, 27, 28]. In all the studies, individuals from sister species did
179 mate, but at least in some cases, heterospecific matings had lower copulation duration than
180 conspecific matings. Our results could represent an early stage of speciation in this regard.
181 Lower copulation duration in BR mating compared to WR mating in M populations could be
182 due to genetic divergence caused by SAC that leads to reduced ejaculate transfer ability
183 and/or cryptic male investment by the males when they mate with allopatric females.

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184 A number of studies - while testing if SAC drives reproductive isolation using experimental
185 evolution - have measured postmating isolation extensively in terms of difference in
186 fecundity[17], offspring number[19, 20], offspring viability[17, 18] or offspring sterility[18], but found no
187 evidence of isolation in those traits. While important, most of them are measures of post-
188 zygotic isolation, which, as these and other studies[7] suggest, are less likely to manifest within
189 tens of generations of selection. Therefore, we focused primarily on prezygotic isolation. An
190 important measure of prezygotic isolation is competitive fertilisation success[8] which none of
191 the studies thus far has addressed. We found that M males have lower competitive
192 fertilisation success when competition happens in BR females than when it does in WR
193 females, while in F males there is no such difference. Since in these populations we could not
194 assay sperm competition directly between BR and WR males due to the lack of phenotypic
195 markers, we have used a proxy measure where all the competitor males used in these assays
196 were taken from the same ancestor population with the assumption that relative sperm
197 competitive ability of the common competitors do not differ across replicate populations
198 within a regime. This is a valid assumption since in a previous study comparing sperm
199 competitive ability of M and F males (where we used the same common competitors) we
200 found no replicate effect [23].

201 There are at least two reasons why M males have reduced sperm competitive ability when
202 mated with allopatric M females. First, it could be a direct correlate of decreased copulation
203 duration. Males with lower copulation duration do not/cannot transfer as much ejaculate and
204 therefore have lower competitive ability[29]. The copulation duration-competitive ability
205 correlation has been demonstrated in the ancestral population from which the selected
206 populations have been derived[25]. Second, it could be a putative stage of conspecific sperm
207 precedence (CSP) –where sperm of conspecific male has greater competitive success over
208 that of heterospecific male. Evidence of CSP is widespread across various taxa[30, 31, 32, 33] and
209 its mechanisms have been illustrated for at least one set of Drosophila sibling species[32, 33]. In

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210 *Drosophila melanogaster* (as in most promiscuous species) females mate multiple times and
211 often store ejaculate (in specialized storage organs, e.g., seminal receptacle and spermatheca
212 in fruit flies) from different males where they compete for fertilisation success. The outcome
213 is mostly determined by how the resident ejaculate (from an earlier mating) is displaced from
214 female storage organs by ejaculate from more recent mating[34] and is influenced by competing
215 males and host female[35]. This provides ample scope for sperm-female coevolution[36]. Since at
216 least some accessory gland proteins are harmful to females, ejaculate- female coevolution
217 should be antagonistic in nature. It is possible that such postmating SAC drove divergence in
218 replicate M populations in terms of how ejaculate and female reproductive tract interact to
219 determine fertilisation success, leading to an incipient form of CSP. Thus, our results show
220 higher rates of SAC can drive reproductive isolation in allopatric populations through
221 reduced postmating competitive success of males.
222 Out of all the studies that have used experimental evolution to test the theoretical prediction
223 that sexually antagonistic coevolution can drive reproductive isolation, there are only two
224 (including the present one) that provide evidence in support, and to the best of our
225 knowledge, this is the only one that provides evidence of postmating isolation. There are
226 multiple reasons as to why our results differ from most of its predecessors[16, 17, 18, 19, 20]:

227 a. The census population size for each replicate was bigger in our study than those of the
228 previous ones.
229 b. The number of generations in those studies were too low (our assays were done after
230 ~100 generations of selection compared to that of ≤ 50 in all of the previous studies)
231 to allow SAC to drive population divergence to a degree where they are apparent.
232 c. According to theoretical predictions, reproductive isolation in allopatric populations is
233 one of the six possible outcomes of sexual conflict[9]. It is possible that the populations
234 under high SAC in those studies did not diverge with respect to each other. However,

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235 none of the studies shed light upon any of the other five possibilities that might have
236 occurred in their populations.

237 In conclusion, we show direct evidence of evolution of both premating and postmating
238 prezygotic RI as a consequence of SAC. Thus, it remains a distinct possibility that sexual
239 conflict can result in a coevolutionary chase between the sexes[11, 37] and can indeed be 'an
240 engine of speciation'. We speculate that initial genetic variation and number of
241 generations can be important to realize – at least in experimental evolution studies –the
242 evolution of RI caused by sexual conflict. However we also feel the need of more such
243 studies to experimentally determine the exact conditions under which sexual conflict
244 leads to reproductive isolation and to elucidate the underlying proximate mechanisms.

245 **Methods:**

246 **Ancestral Populations:**

247 LH – It is a large laboratory adapted population of *Drosophila melanogaster,* established by,
248 and named after Lawrence G. Harshman. The population is maintained on a 14 day discrete
249 generation cycle, under 25°C, 60-80% relative humidity, 12 hours light / 12 hours dark
250 (12hrs: 12hrs L/D cycle) and on standard cornmeal – molasses – yeast food. The flies are
251 grown under moderate larval density of 140-160 per 8-dram vial (25mm diameter × 90mm
252 height) containing 8-10ml food. On the 12th day post egg collection, flies from different vials
253 are mixed and redistributed across fresh food vials containing limiting amount of live yeast
254 grains with 16 males and 16 females per vial. On the 14th day, flies are transferred to fresh
255 vials and are allowed a window of 18 hours to lay eggs which (after discarding the adults and
256 controlling density) start the next generation[38].

257 LH_{st} – This population was derived by introducing the scarlet eye colour (recessive,
258 autosomal and benign) gene into the LH population, hence the subscript. LH_{st} is maintained

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259 under the same condition as LH with N_e>2500. The genetic backgrounds of these two
260 populations are homogenized by periodic back crossing.

261 **Selection Regimes:**

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

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

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

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

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

277

278 **Standardisation and Generation of Experimental Flies:**

279 In order to equalise the potential non-genetic parental effects across different regimes, we
280 maintained all populations under ancestral condition which does not include virgin collection
281 and sex ratio alteration- essentially following the same life cycle as LH_{st} populations for one

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282 generation before obtaining individuals for the experiment. This process is called
283 standardisation[39].

284 Eggs laid by the standardised flies were collected at a density of 150 (±2) per vial (containing
285 8-10ml of cornmeal food) to obtain the experimental flies. On the 10th day after egg
286 collection, males and females were collected as virgins during the peak of their eclosion and
287 held as single individual per vial.

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

292

293 **General Experimental Design:**

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

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

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

304 **Assay for Assortative Mating:**

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305 We combined a virgin female with two virgin males from the same selection regime –one
306 WR and one BR – in vials containing fresh food. That is, a female from a given replicate
307 number was combined with a male from the same replicate number and another from a
308 different replicate number (all within the same selection regime), e.g., one M_1 female + one
309 M_1 male + one M_2 male and so on. Thus, we had three combinations within each selection
310 regime, denoted by female replicate number. Males were marked by pink or green Day-Glo
311 dust for identification. Previous studies using the same dust found no effect on individuals in
312 terms of mating behaviour or female preference [40]. However, to account for any mating bias
313 brought about solely by green and/or pink coloration, we had reverse coloration treatments
314 for all combinations. Thus, each combination had two treatments, e.g., one M_1 female + one
315 green M_1 male + one pink M_2 male; one M_1 female + one pink M_1 male + one green M_2 male
316 and so on. We had 30 replicate vials per combination per colour treatment (Table1). In some
317 vials we observed no mating till one hour after combining the flies. Those vials were
318 discarded and excluded from analysis (Number of discarded vials: 4, 4, and 2 out of 60 trials
319 each from the three replicates in the F regime; 4, 7, and 6 out of 60 trials each from the three
320 replicates in the M regime).

321 **Assay for Mating Latency and Copulation Duration:**

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

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330 **Assay for Competitive fertilisation success:**

331 As a measure of competitive fertilisation success, we measured sperm defense ability of
332 males, the rationale for which is provided in the results section. For assaying sperm defense
333 ability, we set up crosses following the same method as mentioned above and the vials were
334 observed for mating for one hour. The females that did not mate with the first male were
335 discarded. After the first mating, we sorted the females using light CO_2-anaesthesia and held
336 them back into the vials and discarded the males. After allowing a recovery time (from
337 anesthesia) of half an hour, we introduced a second male (red eyed, LH) in each vial and kept
338 the vials undisturbed for 24 hours, during which they could mate with the females. After this
339 exposure window, the second males were discarded and the females were transferred singly
340 (under light anesthesia) to test tubes (dimensions: 12 mm diameter × 75 mm length)
341 provisioned with food. There they were allowed an oviposition window of 18 hours. The
342 adult progeny emerging from the eggs laid during this window were scored for their eye
343 colour marker after 12 days. The proportion of scarlet progeny was taken as an estimate of P1
344 of the male. 90 males from each of the crosses were assayed for P1. Since we did not observe
345 the second mating, instances where all progeny was sired only by the first male (P1=1) could
346 arise due to second male failing to mate. Such instances were excluded from the analysis.
347 Final sample size for P1 analysis was n=83-87 and 70-73 per cross type (WR/BR) in F and M
348 populations respectively. P1 value from a single vial was used as the unit of replication.

349

350 **Statistical Analysis:**

351 To test for assortative mating, we used logistic regression using a mixed model in a nested
352 structure. In the model, the successful mating by a WR male was used as the response
353 variable, selection regime was used as a fixed effect, and replicate population (to which the

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354 female belonged) nested within selection regime was used as a random effect using the
355 following model:

356 WR_Success ~ Selection +(Selection|Block), family=binomial(logtit)

357 The 'glmer' function in the 'lme4' package[42] in R[43] with binomial (logit) family was used for
358 the analysis.

359 For the rest of the assays, we performed a two-way ANOVA with selection regime and
360 treatment (type of individuals involved in a cross: BR/WR) as fixed factors and male and
361 female replicate nested within selection regime as random effects using the following model:

362 response ~ Sel.Reg*Mating.Type +(1|Female.Replicate) + (1|Male.Replicate)

363 In case of significant Sel.Reg*Mating.Type interaction, we performed Tukey's HSD (with α
364 $= 0.05$) for post-hoc analysis. Linear model fitting, ANOVA and post-hoc tests were
365 performed in R using packages 'lme4', 'lmerTest'[44] and 'lsmeans'[45] respectively.

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473

474 **Author Contributions:**

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475 SZA designed the study, carried out experiments, analysed data and wrote the manuscript.

476 MC and MAS carried out experiments. NGP designed the study, and wrote the manuscript.

477 All authors reviewed the manuscript.

478 **Additional information:**

479 **Competing financial interest:** The authors declare that they have NO competing financial interest.

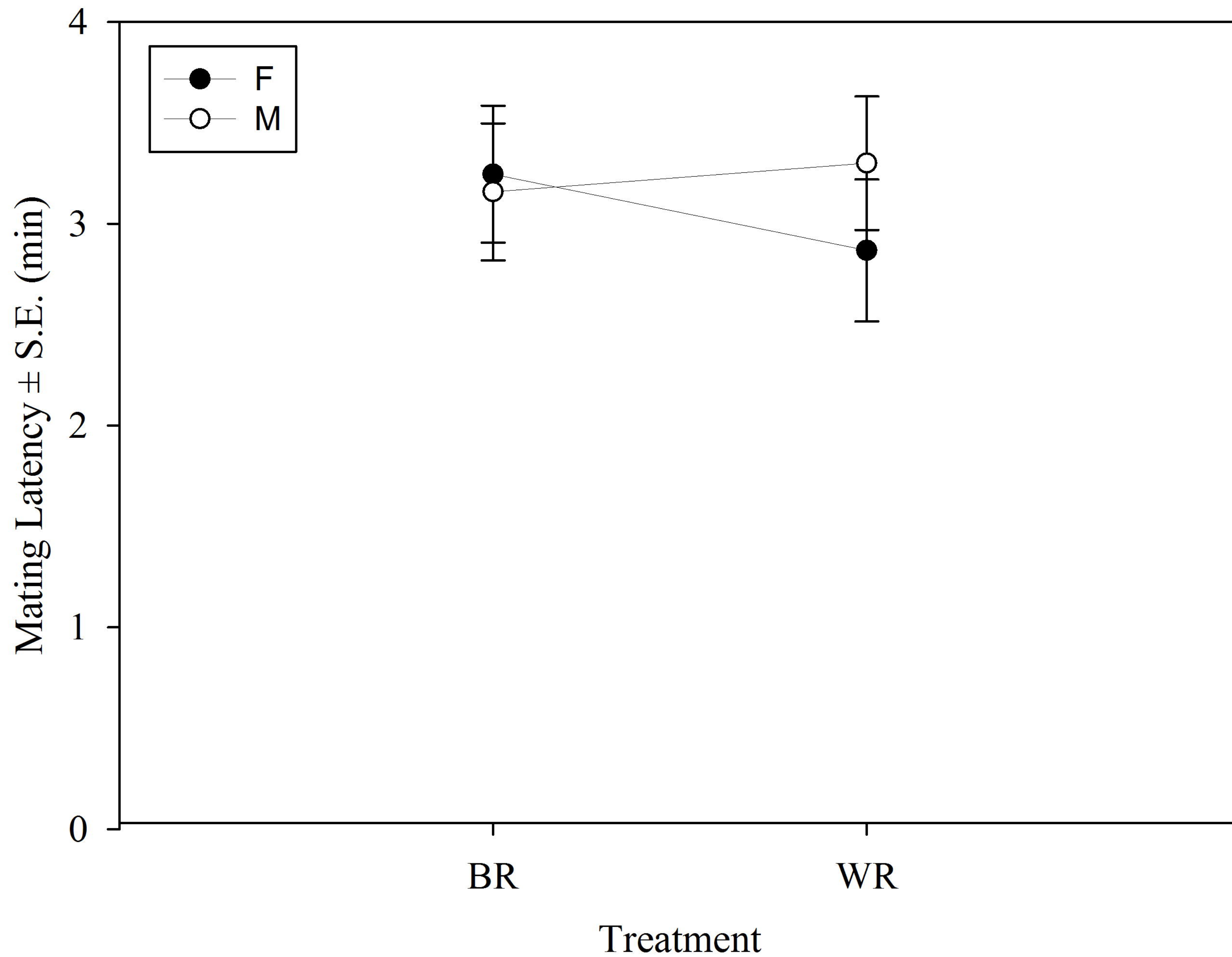
480

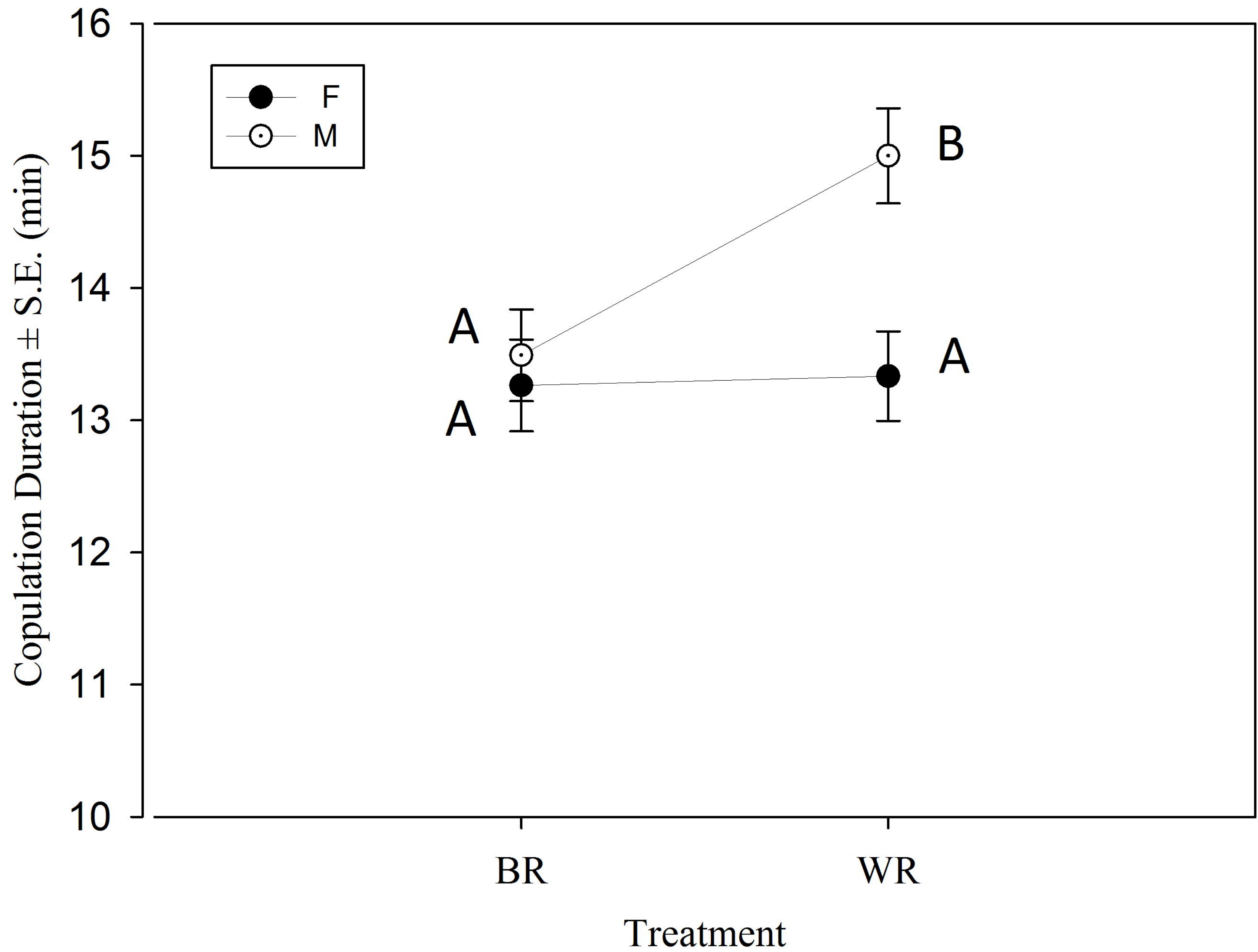
481 **Figure 1: Mean mating latency (±S.E) of WR and BR treatments from female biased (F)**
482 **and male biased (M) regimes based on the results of two-way ANOVA.** There was no
483 significant Selection Regime × Treatment interaction.

484 **Figure 2: Mean copulation duration (±S.E) of WR and BR treatments from female**
485 **biased (F) and male biased (M) regimes based on the results of two-way ANOVA.**
486 Points not sharing common letter (e.g., A and B) are significantly different based on Tukey's
487 HSD.

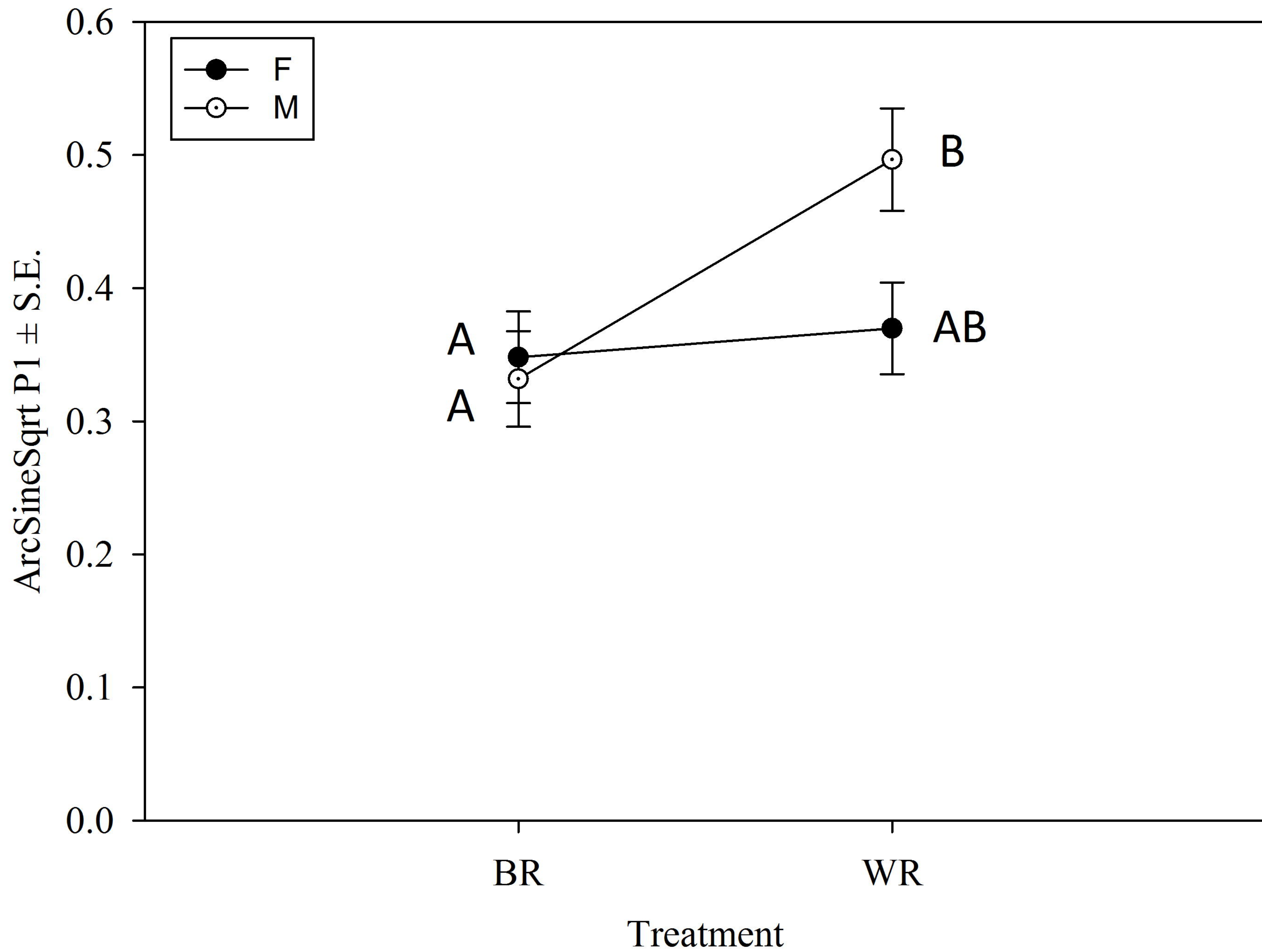
488

489 **Figure 3: Mean (arcsine square root transformed) P1 (±S.E) of WR and BR treatments**
490 **from female biased (F) and male biased (M) regimes based on the results of two-way**
491 **ANOVA.** Points not sharing common letter (e.g., A and B) are significantly different based
492 on Tukey's HSD**.**


F
M
Mating Latency ± S.E. (min)
0
1
2
3
4
BR
WR
Treatment



Copulation Duration ± S.E. (min)
16
15
14
13
12
11
10
F
M
A
A
B
A
BR
WR
Treatment



0.6
0.5
0.4
0.3
0.2
0.1
0.0
ArcSineSqrt P1 ± S.E.
F
M
A
A
B
AB
BR
WR
Treatment