

Reinforcement alone does not explain increased reproductive isolation in sympatry

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Classification

Biological Sciences. Evolution

Keywords

adaptation, *Bufo*, *Drosophila*, Lepidoptera, natural selection, speciation

Author Contributions

DRM designed the research, analyzed and interpreted the data, wrote the paper, and coordinated the study. BSC designed the research, contributed to data analysis and interpretation, and wrote the paper.

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Main Text

Figures 1 to 3

1 **Abstract**

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3 Comparative studies of reproductive isolation (RI) by Coyne and Orr (1, 2) and others
4 indicate a significant role for reinforcing natural selection in *Drosophila* speciation. The
5 reinforcement hypothesis predicts increased prezygotic, but not postzygotic, RI between
6 sympatric species pairs in response to maladaptive hybridization. We revisit this
7 hypothesis and others using additional *Drosophila*, Lepidopteran, and toad (*Bufo*) data.
8 In contrast to the predictions of reinforcement, we find increased premating and
9 postzygotic RI between sympatric *Drosophila* species, including between recently
10 diverged species pairs, as defined by Coyne and Orr (1) and others (i.e., $D_{\text{Nei}} < 0.5$).
11 However, at slightly lower divergence thresholds increased postzygotic RI in sympatry is
12 not statistically significant, while increased premating RI is, generally in agreement with
13 the predictions of reinforcement. While premating data are unavailable, postzygotic RI is
14 also increased between sympatric Lepidopteran and toad (*Bufo*) species. We find only
15 modest support for “concordant asymmetries” in premating and postzygotic RI between
16 sympatric *Drosophila*, described by others as uniquely supporting reinforcement.
17 Finally, the proportion of geographic range overlap shared by species is positively
18 associated with the magnitude of premating RI as predicted by reinforcement, but it is
19 also positively associated with postzygotic RI, which cannot be explained by
20 reinforcement. Taken together, our results demonstrate that comparisons of premating
21 and postzygotic RI in sympatry depend greatly on divergence time, and suggest that
22 fusion, extinction, and/or other mechanisms must combine with reinforcement to
23 generate these patterns.

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32 **Significance Statement**

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34 Understanding mechanisms underlying the origin of species remains a central goal of
35 biology. By combining laboratory estimates of premating and postzygotic reproductive
36 isolation (RI) with phylogenetic hypotheses, geographic range data, and genetic
37 divergence estimates, the fundamental meta-analyses of Coyne and Orr provided
38 compelling support that reinforcement contributes significantly to *Drosophila* speciation.
39 Using additional *Drosophila* data, we demonstrate increased premating and postzygotic
40 RI in sympatry. Postzygotic RI is also increased in sympatry between Lepidopteran and
41 toad species. We also find only modest support for patterns previously described as
42 uniquely supporting reinforcement. Together, our analyses demonstrate that
43 comparisons of premating and postzygotic RI depend on divergence time, and suggest
44 that fusion, extinction, and/or other mechanisms combine with reinforcement to produce
45 these patterns.

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49 Introduction

50 Speciation, the process in which one species splits into two, results from the
51 accumulation of genetic differences and the evolution of reproductive isolation (RI) (3,
52 4). In broad terms, barriers to gene flow can be classified based on when they occur in
53 the reproductive cycle, commonly in relation to the generation of a fertilized zygote (5,
54 6). These barriers fall into two general categories: prezygotic and postzygotic RI.
55 Prezygotic RI includes all ecological, behavioral, mechanical, and gametic
56 incompatibilities that occur before a zygote is formed (5), with most focus placed on
57 premating traits (e.g., mate discrimination). Postzygotic barriers occur after a hybrid
58 zygote is formed and include phenotypes as extreme as hybrid sterility and inviability (7,
59 8), but also more nuanced traits, such as hybrid behavioral defects (9–11) or delays in
60 development (12–14). Coyne and Orr [(1, 2); hereafter “C&O”] completed the first in-
61 depth meta-analyses of speciation that combined estimates of genetic divergence,
62 premating RI, and hybrid viability and fertility in *Drosophila* with phylogenetic and
63 geographic information. Their work demonstrated that both premating and postzygotic
64 RI become stronger as the genetic distance between species increases, and that
65 premating RI accumulates faster in sympatry.

66 Comparative analyses of speciation have greatly influenced our understanding of
67 speciation (15). Analyses similar to those of C&O, and in some cases similar datasets,
68 have revealed seminal aspects of how speciation proceeds. Studies of the rates of
69 evolution of premating and postmating RI between *Drosophila* species pairs have
70 enabled us to understand the role of sex chromosomes in speciation (16–18), and the
71 potential drivers of speciation (19–22). Indeed, C&O sparked a cottage industry to study
72 RI in different groups which has demonstrated that the monotonic increase in RI as
73 divergence accrues is not specific to *Drosophila*, occurring in virtually all studied taxa
74 (23, 24).

75 Arguably the most influential finding of C&O was that in contrast to postzygotic
76 RI, mate discrimination is more pronounced between sympatric than between allopatric
77 species pairs of the same age. These results were taken as evidence that natural
78 selection favors the evolution of premating RI when allopatric species come into
79 secondary contact. In this process, coined reinforcement, premating RI is strengthened

80 as a byproduct of natural selection against hybridization (25–27). These analyses and
81 others led to the conclusion that reinforcement is common during speciation (16, 28). As
82 C&O (1, 2) noted, “any factor that reduces gene flow should inhibit fusion or extinction”.
83 Observing increased prezygotic *and* postzygotic RI in sympatry, particularly for recently
84 diverged species, would support a fusion and/or extinction hypothesis since only
85 populations separated by strong mating discrimination may persist (1, 29, 30).

86 Additional premating and postzygotic RI estimates from *Drosophila*, in
87 combination with estimates of postzygotic RI between Lepidopteran and between toad
88 (*Bufo*) species, provide the opportunity to revisit and test the predictions of
89 reinforcement and related hypotheses. First, and in contrast to C&O, we find increased
90 premating and postzygotic RI between recently diverged sympatric relative to allopatric
91 *Drosophila* pairs using their divergence threshold ($D_{\text{Nei}} < 0.5$); but at slightly lower
92 thresholds increased postzygotic RI in sympatry is not statistically significant, while
93 increased premating RI is, in agreement with the predictions of reinforcement. Second,
94 while butterfly and toad data do not include estimates of premating RI, we also observe
95 increased postzygotic RI in sympatry for these taxa, including for the most recently
96 diverged toads. Third, our expanded *Drosophila* analyses find only modest evidence for
97 stronger premating RI among the more costly reciprocal matings. Fourth, premating RI
98 is positively associated with the proportion of geographic range overlap, but so is
99 postzygotic RI, which cannot be explained by reinforcement. Our results suggest that
100 fusion, extinction, and/or other mechanisms combine with reinforcement to generate the
101 patterns we observe.

102

103 **Results**

104

105 ***Increased premating and postzygotic RI in sympatry***

106 RI increases as species diverge, and several efforts have reported a negative
107 correlation between parental species divergence and both the strength of mating
108 discrimination (1, 2, 31) and the fitness of resulting hybrids (e.g., (32–34), reviewed in
109 (15, 23, 24, 35)). We fit three types of models to study the evolution of RI between
110 diverging species. For both premating and postzygotic RI in *Drosophila*, four-parameter

111 logistic (henceforth abbreviated 4PL) models fit the data better than do linear or logistic
112 models, for both allopatric and sympatric species pairs (Table S1). The 4PL model was
113 also the best fit for postzygotic RI in toads and Lepidopterans, for both allopatric and
114 sympatric species pairs. The 4PL model meets the biological expectation of a waiting
115 time for mutations that generate either hybrid incompatibility (36–38) or behavioral
116 isolation (31), followed by a rapid accumulation of RI that eventually asymptotes. Please
117 note that even though Nei's D , our metric of genetic differentiation between *Drosophila*
118 parental species, is not a perfect predictor of the extent of differentiation, it represents a
119 good proxy (Figure S1 in (9)).

120 Next, we re-assessed support for the hypothesis that reinforcement has driven
121 the evolution of premating RI in sympatry for *Drosophila*. We leveraged the information
122 provided by our model fits and compared the values of the lower asymptote, the upper
123 asymptote, and the inflection point for sympatric and allopatric species pairs, for both
124 premating and postzygotic RI (Figure 1A and B, respectively). Table S2 shows pairwise
125 comparisons for the regression parameters for allopatric and sympatric species. To
126 determine whether isolation evolved faster in sympatric species, we compared c , the
127 inflection point of the 4PL model, and a proxy for how fast RI completes, for regressions
128 using either sympatric or allopatric species. Consistent with previous studies (1, 2)
129 premating RI between sympatric *Drosophila* species pairs reaches c earlier than
130 allopatric species pairs ($C_{\text{Sympatric-}Drosophila\text{-premating}} = 0.059$; $C_{\text{Allopatric-}Drosophila\text{-premating}} = 0.318$;
131 Wilcoxon rank sum test with continuity correction: $W = 978,080$, $P < 1 \times 10^{-10}$; Figure
132 1C). The rate of increase once the inflection point is reached (Hill's slope, or b) is higher
133 for allopatric pairs which eventually leads to similar d (asymptote) values. In Figure S1,
134 we present the other regression parameters: a , b , and d . Surprisingly, we observed that
135 for postzygotic RI, c is lower and b is higher for sympatric than for allopatric pairs,
136 indicating that postzygotic RI also increases faster as divergence accrues between
137 sympatric species ($C_{\text{Sympatric-}Drosophila\text{-postzygotic}} = 0.439$; $C_{\text{Allopatric-}Drosophila\text{-postzygotic}} = 0.751$;
138 Wilcoxon rank sum test with continuity correction: $W = 993,050$, $P < 1 \times 10^{-10}$; Figure
139 1D). These results suggest that both premating and postzygotic RI increase faster
140 between sympatric than between allopatric species pairs. We find similar results for
141 both premating and postzygotic RI in *Drosophila* after using stringent subsamplings at

142 the species-group level (Premating: $c_{\text{Sympatric-Drosophila}} = 0.141$; $c_{\text{Allopatric-Drosophila}} = 0.394$, W
143 $= 16,917$; $P = 3.654 \times 10^{-11}$; Postzygotic: $c_{\text{Sympatric-Drosophila}} = 0.38$; $c_{\text{Allopatric-Drosophila}} =$
144 0.610 , $W = 8,342$; $P = 3.613 \times 10^{-10}$; Figure S2).

145 Next, we fit a phylogenetically informed linear regression. The models included
146 an effect for geographic origin and an interaction between origin and genetic distance. If
147 RI evolves similarly in sympatry and allopatry, as expected for postzygotic RI under a
148 scenario of pure-reinforcement driving the evolution of strengthened RI in sympatry,
149 then effects of both origin and the interaction should be negligible. For premating RI, we
150 found that sympatric species display generally stronger premating RI than do allopatric
151 species (95% CI=[0.245, 0.464], $P < 0.001$), but the increase of premating RI is slower
152 in sympatric species (95% CI = [-0.323, 0.058], $P = 0.014$; Table S3). For postzygotic
153 RI, we found that sympatric and allopatric species have a similar level of postzygotic RI
154 (95% CI = [-0.121, 0.198], $P = 0.660$), but that postzygotic RI increases faster with
155 genetic distance in sympatry (95%CI = [0.138, 0.699], $P = 0.002$; Table S3). These
156 results are consistent with previous reports of stronger RI in sympatry using a linear
157 model of accumulation of premating and postzygotic RI (39). They are also consistent
158 with the results from the uncorrected datasets, demonstrating that in *Drosophila*
159 premating and postzygotic RI both evolve faster in sympatry.

160 While premating data are not available, we also studied these same parameters
161 for postzygotic RI between Lepidopteran species and *Bufo* toads (Figure 2). The 4PL
162 regression results for these taxa are similar to those in *Drosophila* (Figure 2A and B).
163 The inflection point is lower for sympatric than for allopatric species pairs for both
164 Lepidopterans ($c_{\text{Sympatric-Lepidopterans}} = 0.686$; $c_{\text{Allopatric-Lepidopterans}} = 0.834$, $W = 89,102$, $P <$
165 1×10^{-10} ; Figure 2C) and *Bufo* toads ($c_{\text{Sympatric-Toads}} = 0.028$; $c_{\text{Allopatric-Toads}} = 0.038$, $W =$
166 $130,280$, $P < 1 \times 10^{-10}$; Figure 2D). For Lepidopterans, b is much higher for sympatric
167 than for allopatric species pairs ($b_{\text{Sympatric-Lepidopterans}} = 242.1179$; $b_{\text{Allopatric-Lepidopterans}} =$
168 7.321 , $W = 130,280$, $P < 1 \times 10^{-10}$; Figure S3), which reflects that substantial
169 postzygotic RI is rare between allopatric species pairs even in the most divergent
170 crosses (i.e., *Papilio xuthus* \times *P. glaucus* being an exception, Nei's $D = 1.161$).
171 Subsampling by genus produces a similar result. While c in sympatric species is similar
172 to the complete dataset ($c_{\text{Sympatric-Lepidopterans}} = 0.887$), b is lower but positive ($b_{\text{Sympatric-}}$

173 Lepidopterans = 5.801). Neither of these two parameters could be calculated for allopatric
174 species because there was no increase in the magnitude of postzygotic RI over genetic
175 distance for these species, and the 4PL regressions did not converge.

176 For *Bufo*, b is higher for allopatric than for sympatric species pairs ($b_{\text{Sympatric-Toads}}$
177 = 1.734; $b_{\text{Allopatric-Toads}}$ = 2.899, $W = 26,916$, $P < 1 \times 10^{-10}$; Figure S3), which is consistent
178 with the earlier inflection point for sympatric pairs and the similar asymptote of both
179 cases ($d = 1$, Figure S3). A subsampling by subgenus was not possible for *Bufo* (See
180 Methods).

181 We followed up the analyses in these two groups with a phylogenetically
182 corrected linear regression. In the case of Lepidopterans, sympatric species have
183 slightly higher postzygotic RI compared with allopatric species (95% CI = [-0.018,
184 0.257], $P = 0.076$), similar to premating RI in *Drosophila*. The increase of inviability with
185 genetic distance is faster for sympatric species than for allopatric species (95% CI = [-
186 0.674, -0.094], $P = 0.014$; Table S4). In *Bufo*, we find that postzygotic RI is generally
187 lower in sympatric species (95% CI = [-0.272, -0.032], $P = 0.006$), but that RI increases
188 faster with genetic distance between sympatric species pairs (95% CI = [0.486, 3.637],
189 $P = 0.012$; Table S4). These regressions are more limited than 4PL regressions, as
190 linear regressions do not differentiate between the inflection point (c) and the rate of
191 increase with genetic distance (b). Nonetheless, these results are consistent with the
192 4PL results in that the rate of evolution of postzygotic RI is not equivalent in allopatric
193 and sympatric species.

194 In sum, our results indicate that across the full range of divergence both
195 premating and postzygotic RI are stronger between sympatric than allopatric species for
196 three different animal taxa. Because analysis of RI across the full range of divergence
197 may conflate conditions for co-occurrence of reproductively isolated lineages with the
198 conditions that accelerate speciation, we next assessed RI for the most recently
199 diverged species.

200

201 ***Comparisons of RI in sympatry depend on divergence time***

202 We first restricted our dataset to recently diverged *Drosophila* pairs as defined by
203 C&O ($D_{\text{Nei}} < 0.5$) to test if premating, but not postzygotic, RI is strengthened at earlier

204 stages of divergence. Both pre mating ($W = 479.5$; $P < 0.0001$) and postzygotic ($W =$
205 902.5 , $P = 0.039$) RI are stronger in sympatry using the C&O divergence threshold for
206 young species. However, this result is highly contingent on the arbitrary choice of how
207 to define recently diverged species, such that with a slightly lower threshold ($D_{\text{Nei}} =$
208 0.47) the strength of postzygotic RI in sympatry and allopatry does not differ ($W = 881.5$,
209 $P = 0.05$; Figure S4). In contrast, postzygotic RI between toads is always significantly
210 increased in sympatry relative to allopatry (Table S5). Fitting 4PL regressions to the
211 most recently diverged *Drosophila* ($D_{\text{Nei}} < 0.5$) and *Bufo* pairs (NJ distance < 0.05 ;
212 Figure S5) indicates that the inflection point of the regression occurs earlier for
213 sympatric than for allopatric *Drosophila* ($c_{\text{sympatric}} = 0.366$; $c_{\text{allopatric}} = 0.468$; $W = 812,777$;
214 $P < 0.0001$) and *Bufo* ($c_{\text{sympatric}} = 0.029$; $c_{\text{allopatric}} > 0.5$) pairs. Similar analyses were not
215 possible for Lepidopterans because the 4PL regressions did not converge when the
216 dataset included only species pairs with Nei's $D < 0.5$.

217 In sum, our results indicate that comparisons of pre mating and postzygotic RI
218 between sympatric *Drosophila* depend greatly on divergence time; and while
219 comparisons that include only the most recently diverged *Drosophila* species support
220 the predictions of reinforcement, elevated postzygotic RI between sympatric *Drosophila*
221 and *Bufo* at relatively early stages of divergence implies other mechanisms also
222 contribute to these patterns.

223

224 **Proxies of the cost of hybridization are correlated with the strength of pre mating** 225 **and postzygotic RI in *Drosophila***

226 We next tested three additional predictions of reinforcement as the primary driver
227 of strengthened pre mating RI in sympatry. First, if pre mating RI evolves via
228 reinforcement, then the selection pressure to evolve pre mating RI might be a function of
229 the cost of hybridization (40, 41). It then follows that if the strength of RI differs between
230 reciprocal crosses, the cross direction with stronger postzygotic RI, and thus a higher
231 cost to hybridization, should also display stronger pre mating RI (42). A pattern of
232 'concordant asymmetries' (i.e., asymmetries in pre mating and postzygotic RI in the
233 same direction) has been observed in selected sympatric *Drosophila* species (16

234 species pairs), but not in allopatric ones (20 species pairs), which is argued to uniquely
235 support the prevalence of reinforcement ((42) but see (16)).

236 We extend this argument to the strength of asymmetries (i.e., cross directions
237 with strong postzygotic RI should show relatively strong premating RI), testing for a
238 positive correlation between the magnitude of premating and postzygotic RI
239 asymmetries in *Drosophila*. Across all of the data, the strength of the asymmetry in
240 postzygotic RI predicts the strength of the asymmetry in premating RI in sympatry ($N =$
241 73 , $F_{1,71} = 8.037$, $P = 0.006$), but not in allopatry ($N = 91$, $F_{1,89} = 0.002$, $P = 0.964$), but
242 the proportion of premating RI asymmetry explained by postzygotic RI asymmetry in
243 sympatry is low ($R^2 = 0.089$, Figure 3A). Figure S6 shows that the association between
244 the strength of premating and postzygotic RI asymmetries is sensitive to inclusion
245 parameters in the study. If only cases where the magnitude of postzygotic RI is
246 asymmetric are included, the association between the magnitude of premating and
247 postzygotic RI asymmetries is not significant (sympatry: $F_{1,11} = 1.381$; $P = 0.265$;
248 allopatry: $F_{1,16} = 1.905$, $P = 0.186$; dashed lines in Figures 3A). A partial correlation
249 while controlling for genetic distance shows that the magnitude of premating and
250 postzygotic RI asymmetries are only weakly correlated ($\rho = 0.192$, $P = 0.050$). Thus,
251 even if concordant asymmetries are taken as incontrovertible evidence for
252 reinforcement (see below), we interpret these results as supporting that other
253 mechanisms also contribute to strengthened RI between sympatric *Drosophila* species.

254 Second, because the risk of hybridization is proportional to the extent of
255 geographic range overlap, in sympatry reinforcement should produce a positive
256 relationship between the strength of premating RI between species and the proportion
257 of their range overlap. This relationship should not exist for postzygotic RI since there is
258 no impetus for reinforcement to act on it (1). Consistent with the reinforcement
259 hypothesis and findings of previous reports (43), we find that range overlap is positively
260 associated with premating RI in *Drosophila*, even after controlling for genetic distance
261 (Linear Model: $F_{1,255} = 50.809$, $P = 1.044 \times 10^{-11}$; Figure 3B). However, the proportion of
262 geographic range overlap is also positively associated with the magnitude of postzygotic
263 RI in *Drosophila* ($F_{1,105} = 14.098$, $P = 2.85 \times 10^{-4}$; Figure 3C). Table S6 shows the
264 results for the linear models. Notably, there is no relationship between geographic range

265 overlap and the strength of premating or postzygotic RI in species whose ranges
266 overlap more than 5%, suggesting the *Drosophila* result is driven by species pairs that
267 are mostly allopatric (Table S7). This result suggests that in some instances overlap
268 might drive the evolution of both premating and postzygotic RI, and is thus inconsistent
269 with the predictions of reinforcement being the exclusive driver of premating RI in
270 sympatry.

271 Finally, and as C&O (1) noted, if reinforcement is responsible for the
272 strengthened RI in sympatry, then sympatric pairs should display a range of RI values
273 that is higher and outside the range observed between allopatric pairs because natural
274 selection will lead to RI after secondary contact. Conversely, if differential fusion and/or
275 extinction is responsible for the pattern of stronger RI in sympatry (29, 30), the range of
276 RI observed among sympatric pairs should be a subset of the RI observed among
277 allopatric pairs because species pairs with weak RI will go extinct (1). The range of RI
278 among sympatric pairs is similar to that of allopatric pairs (i.e., both are bounded by 0
279 and 1), including with the Coyne and Orr (1) threshold for young species (Nei's $D < 0.5$;
280 Figure S7). (Please note that the range is not equivalent for *Drosophila* species pairs
281 younger than Nei's $D < 0.25$ ((42); see Discussion). Sympatric and allopatric *Bufo* pairs
282 show a similar range of postzygotic RI regardless of divergence, but this is not the case
283 for Lepidopterans (Figure S8). We interpret these results as indicating that multiple
284 mechanisms likely contribute to increased premating RI in sympatry.

285

286 **Discussion**

287 Comparative studies are a powerful tool to study how RI accrues, divergence
288 proceeds, and speciation occurs. Groundbreaking analyses by C&O and others broadly
289 support the hypothesis that reinforcement contributes significantly to *Drosophila*
290 speciation, but as C&O (1) noted “enhanced prezygotic RI could result from a process
291 of fusion or extinction in sympatry” because “any factor that reduces gene flow should
292 inhibit fusion or extinction” (pg. 376). Such a mechanism could produce higher
293 premating RI in sympatry, in the absence of reinforcement (29). We discuss several
294 lines of evidence that indicate multiple mechanisms likely contribute to increased RI in
295 sympatry.

296 First, both premating and postzygotic RI are increased in sympatry for recently
297 diverged *Drosophila* species as defined by Coyne and Orr (1), but at slightly lower
298 divergence thresholds increased postzygotic RI in sympatry is no longer statistically
299 significant. This pattern generally supports the predictions of reinforcement, while
300 highlighting that comparisons of RI in sympatry depend greatly on divergence time.
301 Postzygotic RI is also increased in sympatry between Lepidopteran and between the
302 most recently diverged *Bufo* species. (Premating data are not available in either
303 system.) Elevated postzygotic RI in sympatry for relatively recently diverged pairs in
304 three divergent taxa implies multiple mechanisms combine to produce these patterns.

305 Second, the absence of very recently diverged and strongly isolated allopatric
306 *Drosophila* species pairs has been argued as support for reinforcement and against
307 fusion and extinction. If differential fusion and extinction contribute to elevated
308 premating RI in sympatry, we should also observe strongly prezygotic isolated pairs in
309 allopatry. In contrast to this prediction, Yukilevich ((42), Figure 1) reported a paucity of
310 very recently diverged allopatric *Drosophila* pairs ($D < 0.25$) with strong premating RI
311 (i.e., a “Templeton gap”). While this result supports the predictions of reinforcement, we
312 argue it must depend on species definitions. For example, multiple instances in the
313 literature report strong assortative mating between populations of the same species
314 (i.e., *D. lacertosa*: Table 2 in (44); *D. yakuba*: Table 4 in (45); and 14 other species in
315 (46)). Researchers are more likely to classify strongly isolated sympatric groups as
316 different taxonomic species since their isolation has been “tested” in secondary contact.
317 This creates an inherent bias when making these comparisons at the early stages of
318 divergence, producing the illusion of a gap. This bias and the existence of strongly
319 isolated allopatric populations within species that are weakly differentiated leads us to
320 conclude that support for the Templeton gap in *Drosophila* is less compelling than
321 previously argued. Notably, including these intraspecific observations lowers the
322 inferred c value for allopatric species pairs, but not enough to change the conclusion
323 that premating RI accumulates faster in sympatric species (Figure S9).

324 Third, asymmetries in postzygotic RI and the extent of geographic range overlap
325 between species pairs have also been proposed as support for reinforcement of
326 premating RI (42). Consistent with previous analyses, we find that in sympatry, sides of

327 reciprocal *Drosophila* crosses that produce strong postzygotic RI also produce strong
328 premating RI; however, asymmetry in postzygotic RI explains only 9% of the variance in
329 premating RI asymmetries. While this pattern might be explained by the predictions of
330 reinforcement, it might also be explained by differential fusion and/or extinction. In
331 instances where premating and postzygotic RI act jointly to prevent fusion (47), one
332 could also expect concordant asymmetries even if reinforcement is not driving the
333 pattern of enhanced RI in sympatry. The same pattern of concordant asymmetries could
334 also be explained by slight asymmetries in gene flow, which could produce
335 asymmetrical accumulation of intrinsic postzygotic RI (16). Given that we also observe a
336 strong effect of geographic range overlap on the evolution of premating RI and
337 postzygotic RI, these results provide further evidence that no single mechanism
338 explains increased RI in sympatry.

339 What then explains our observation of stronger postzygotic RI in sympatry for
340 three divergent taxa? One possibility is that postzygotic RI also evolves through
341 pervasive reinforcing selection. This would explain the faster evolution of premating and
342 postzygotic RI in sympatry and the strong effect of geographic overlap in both barriers.
343 Reinforcement of postmating RI has been demonstrated in *Drosophila* (48, 49) and
344 *Neurospora* (50, 51), but in all cases the reinforced barriers act prior to zygote formation
345 (i.e., postmating-prezygotic barriers). Even though hybrid inviability has been
346 hypothesized to potentially evolve through selection against hybrids (52), reinforcement
347 should only occur for hybrid inviability if aborting the hybrid embryos might represent an
348 advantage to the parents. (No similar rationale has been proposed for hybrid sterility.)
349 Since *Drosophila*, Lepidopterans, and toads all show external development, there is no
350 impetus for selection to reinforce postzygotic RI.

351 A second possibility is that stronger postzygotic RI in sympatry results in part
352 from fusion and/or extinction (29, 30, 53). In cases when postzygotic RI is increased in
353 sympatry, and specifically in cases when both premating and postzygotic RI are
354 stronger in sympatry, differential fusion is a more likely driver of the pattern of enhanced
355 RI in sympatry than reinforcement (1). This idea has been disfavored because C&O did
356 not observe elevated postzygotic RI for recently diverged sympatric *Drosophila* species,
357 and because of the Templeton gap (see above). Our observation of increased

358 postzygotic RI between relatively recently diverged *Drosophila*, butterflies, and between
359 recently diverged toads implies a role for fusion, extinction, and/or other mechanisms.

360 A final, and under-discussed possibility, is that the pattern of stronger RI in
361 sympatry is due to gene flow which might lead to a systematic underestimation of the
362 age of sympatric species. This will disproportionately affect recently diverged sympatric
363 species as the extent of gene flow seems to be negatively correlated with the
364 divergence age between the parental species (54, 55). This possibility might affect our
365 inference of stronger postzygotic RI in sympatry and specifically create the appearance
366 of stronger RI at low genetic distances. We argue this is an unlikely possibility. In the
367 case of *Drosophila*, the vast majority of introgression is purged rapidly (i.e., within the
368 first ten generations) after admixture (56–58). The percentage of the genome that
369 shows evidence of gene exchange among *Drosophila* species pairs ranges between
370 0.1% and 5% (59–61). Nonetheless, the effect of introgression on point measurements
371 of differentiation deserves a systematic treatment.

372

373 **Conclusions**

374 Research from the last three decades has revealed dozens of cases of
375 strengthened RI in sympatry resulting from reinforcement (reviewed in (26–28)).
376 Experimental evolution has also demonstrated that RI can evolve rapidly if selection
377 against hybridization is strong, lending additional support to the possibility of
378 reinforcement in nature (48, 62–65). Using an expanded dataset, our *Drosophila*
379 analyses generally agree with the main finding of C&O that premating, but not
380 postzygotic, RI is increased between the most recently diverged sympatric species
381 pairs. However, the dependence of this result on divergence time, our observation of
382 increased postzygotic RI between sympatric butterflies and toads, and the other lines of
383 evidence discussed above support that fusion, extinction, and/or other mechanisms
384 must combine to produce these patterns.

385 Notably, strengthened RI does not seem to be a deterministic outcome of
386 sympatry. *Glycine* and *Silene* plant pairs show similar levels of postmating-prezygotic RI
387 in sympatry and allopatry (66). A concerted effort to compare the prevalence of
388 strengthened RI in sympatry, and its potential causes across divergent taxa, is sorely

389 needed. We expect that those analyses will reveal more support that no single
390 mechanism underlies elevated RI in sympatry. While comparative studies of RI are
391 useful for testing hypotheses across diverse groups of organisms, the results of our
392 analyses suggest to us that we need a better understanding of the exact mechanisms
393 underlying the evolution of RI in sympatry.

394

395 **MATERIALS AND METHODS**

396

397 **Datasets**

398

399 Our goal was to study whether the rate of evolution of postzygotic RI is similar in
400 sympatric and allopatric animal species pairs. We gathered seven datasets that
401 included metrics of RI (Table S8). Five of these datasets included estimates of genetic
402 distance and the extent of geographic range overlap (i.e., whether species pairs are
403 sympatric or allopatric). Two of these studies have fewer than five species pairs in each
404 category, leaving only three datasets that include systematically gathered metrics of
405 postzygotic RI in sympatric and allopatric species (Table S8). First, we used the data on
406 over 630 *Drosophila*-species pairs from Yukilevich (42), the most extensive compilation
407 of measurements of reproductive RI in *Drosophila*. This compendium included all of the
408 data from C&O (1, 2), data on postzygotic RI across *Drosophila* from Bock (67), and
409 new data collected by Yukilevich (42). In total, the dataset includes 288 interspecific
410 hybridizations with estimates of genetic distance. Of these, 140 species pairs were
411 classified as sympatric and 148 as allopatric. The second dataset was postzygotic RI in
412 Lepidopterans (data from (68)). The dataset includes 212 interspecific hybridizations, 68
413 of which have measurements of genetic distance. Of these, 52 species pairs were
414 classified as sympatric and 16 as allopatric. The final dataset, was postzygotic RI in
415 toads (data from (39, 69)). This dataset includes 669 interspecific hybridizations, all of
416 which have measurements of genetic distance (calculated from a neighbor-joining tree,
417 (39)). Ninety-five species pairs were classified as sympatric and 574 as allopatric. Only
418 the *Drosophila* dataset includes estimates of premating RI. Please note that the
419 measurement of genetic distance between toad species (derived from a neighbor-

420 joining tree) is not equivalent to that in *Drosophila* and Lepidopterans (Nei's *D*), so we
421 refrain from doing among comparison groups between insects and anurans.

422

423 **Model fitting**

424

425 We investigated the relationship between the strength of RI (either premating or
426 postzygotic in *Drosophila*, or postzygotic in Lepidopterans and toads) and the genetic
427 distance between the parental species. First, we compared the fit of three models to the
428 accumulation of RI with genetic distance: linear, logistic increase, and a four-parameter
429 logistic (i.e., dose-response) curve. We fit the linear model using the *lm* (library 'stats')
430 function in R (70) and the other two models using the *nlsLM* (library 'minpack.lm', (71))
431 function in R. The logistic models followed the form:

432

$$433 \quad Isolation \sim \frac{1}{1 + e^{-((a \times Genetic\ distance) + b)}} \quad (1)$$

434

435

436 where *Isolation* is either a premating or postzygotic RI metric, *a* is when the genetic
437 distance is zero, and *b* adjusts how quickly the probability changes with a single-unit
438 change.

439

440 The four-parameter logistic models had the form:

$$441 \quad Isolation \sim d + \frac{a-d}{1 + \left(\frac{Genetic\ distance}{c}\right)^b} \quad (2)$$

442

443 where *a* is the magnitude of isolation in a given cross at the minimum genetic distance
444 (here where Nei's *D* = 0), *b* is the rate of increase in RI at point *c*, the inflection point of
445 the decay curve, and *d* is the maximum RI that can be obtained at high genetic
446 distance. This model allows for an initial period where F1 hybrids do not suffer fitness
447 consequences (34, 38) and includes that RI must asymptote at a value no higher than 1
448 (i.e., more genetic changes contributing to isolation become redundant as two lineages
449 cannot be more isolated than completely isolated). Since nonlinear logistic regression

450 has difficulties optimizing the values for each of the four constants in the equations, we
451 tried 10 starting values per constant and found the model with the lowest Akaike
452 Information Criterion (AIC, (72)) with the function 'AIC', (library 'stats', (70)).

453 To determine which of the four models best describes the relationship between
454 isolation and genetic divergence we used AICs (72). To find the confidence intervals of
455 the logistic regressions we bootstrapped the datasets 1,000 times using the function
456 *nlsBoot* (library('nlstools', (73)). Finally, we compared the confidence intervals using
457 Wilcoxon tests of the bootstrap values for each coefficient using the function *wilcox.test*
458 (library 'stats', (70)).

459

460 **Phylogenetic independence**

461

462 Ideally, our estimates of RI would be phylogenetically independent from all others.
463 However, our *Drosophila* dataset contains multiple species pairs with phylogenetic
464 relationships that are not evolutionarily, and thus might not be statistically, independent.
465 To account for this lack of independence, we used clade-level and species-level
466 sampling schemes for *Drosophila*, and a species-level sampling scheme for *Bufo* frogs
467 (9, 66). For the species group-level sampling scheme in *Drosophila*, we sampled a
468 single cross from within each monophyletic clade of *Drosophila* (*affinis*, *ananassae*,
469 *athabasca*, *buzzatii*, *melanogaster*, *mesophragmatica*, *montium*, *mulleri*, *nasuta*,
470 *obscura*, *planitibia*, *pseudobscura*, *repleta*, *takahashii*, *virilis*, and *willistoni*; Suvorov et
471 al. 2020) resulting in two datasets: one allopatric and one sympatric, each composed of
472 about 16 species pairs. We subsampled the whole dataset 1,000 times and recalculated
473 the value of *c*, the inflection point, for each iteration as described above (See Model
474 fitting). We recorded the number of iterations that did not converge (i.e., the inferred *c*
475 value was outside of bounds of the range of the function, in this case larger than the
476 maximum Nei's *D* value), but restricted comparisons between $c_{\text{Sympatric}}$ and $c_{\text{Allopatric}}$ to
477 regressions that converged. We followed a similar approach for Lepidopterans
478 subsampling by genus (*Anartia*, *Anthocharis*, *Callosamia*, *Choristoneura*, *Colias*, *Erebia*,
479 *Heliconius*, *Helicoverpa*, *Heliiothis*, *Hyalophora*, *Papilio*, *Phyciodes*, *Pieris*, *Pontia*, and
480 *Ypomeneuta*). We did not use this approach for *Bufo* toads because a large proportion

481 of the hybridizations involved species from different *Bufo sensu lato* genera (*Bufo sensu*
482 *stricto*, *Sclerophrys*, *Schismaderma*, *Rhinella*, *Incillius*, and *Anaryxus*).

483 We also formally corrected the data using the phylogenetic mixed model
484 approach proposed by Castillo (39) for the three different taxa. We fitted a generalized
485 linear mixed model using Markov Chain Monte Carlo to study the relationship between
486 RI and genetic distance for sympatric and allopatric species. We used the function *ginv*
487 (library *MASS*, (74)) to find the generalized inverse of the (1-genetic distance) matrix
488 (39). We used the package *MCMCglmm* (75) and fitted a linear model, in which the
489 magnitude of isolation (prematuring or postzygotic for *Drosophila*, and postzygotic for
490 Lepidopterans and *Bufo*) was the response variable, genetic distance was a predictor
491 variable, geographic overlap (i.e., whether a species pair was sympatric or allopatric)
492 was a fixed effect, and the phylogenetic variance matrix was a random effect. The
493 model also included an interaction between transformed genetic distance and
494 geographic overlap. We ran two independent MCMC chains. To determine if the model
495 converged in each of the two chains, we used the function *gelman.diag* (library *coda*,
496 (76)). A chain was considered converged if all scale reduction factors for all variables
497 (both fixed and random effects) were ≤ 1.1 for each of the two chains. We calculated the
498 95% confidence interval for the intercept and the slope using the function *HPDinterval*
499 (library *coda*, (76)).

500

501 **Asymmetries and geographic range**

502 Asymmetries and overlap of geographic range are two proxies of the risk and
503 cost of hybridization. For sympatric species pairs where postzygotic RI is stronger for
504 one of the two reciprocal crosses, premating RI should also be asymmetric and stronger
505 for the same cross direction if reinforcement has driven the evolution of premating RI.
506 This correlation should not exist for allopatric species pairs (42). For the *Drosophila*
507 dataset, we calculated the proportion of the variance in asymmetries in premating RI
508 explained by asymmetries in postzygotic RI as the adjusted R^2 from a linear regression.
509 We fit linear models with the function *lm* (library '*stats*', (70)) where the magnitude of the
510 asymmetry in premating RI was the response, and the magnitude of the postzygotic RI
511 was the only continuous effect ($N = 167$ species pairs). We also calculated the partial

512 correlation coefficient between asymmetry in premating and postzygotic RI while
513 controlling for genetic distance using the function *pcor.test* (*ppcor* library, (77)) for a
514 dataset that included the magnitude of both asymmetries and genetic distance ($N = 106$
515 species pairs).

516 We used a linear model to study the effect of geographic range overlap on
517 patterns of RI. Since we studied two types of RI barriers, we fitted two different linear
518 models. The approach for the two models was identical and the model with the
519 interaction followed the form:

520

$$\begin{aligned} \text{Isolation} \sim & \text{Percentage sympatry}_i + \text{Genetic distance}_j \\ & + (\text{Percentage sympatry} \times \text{Genetic distance})_{ij} + \text{Error}_{ij} \end{aligned}$$

521

522 where *Isolation* refers to either a premating or postzygotic RI metric. To assess the
523 significance of the interaction, we followed a maximum-likelihood model simplification
524 approach (78, 79), in which we compared the model with the interaction to one without
525 the interaction that followed the form:

526

$$\text{Isolation} \sim \text{Percentage sympatry}_i + \text{Genetic distance}_j + \text{Error}_{ij}$$

527

528 To compare the two linear models, we used the function *lrttest* (library '*lmtest*', (80)).

529

530

531 **Acknowledgements**

532

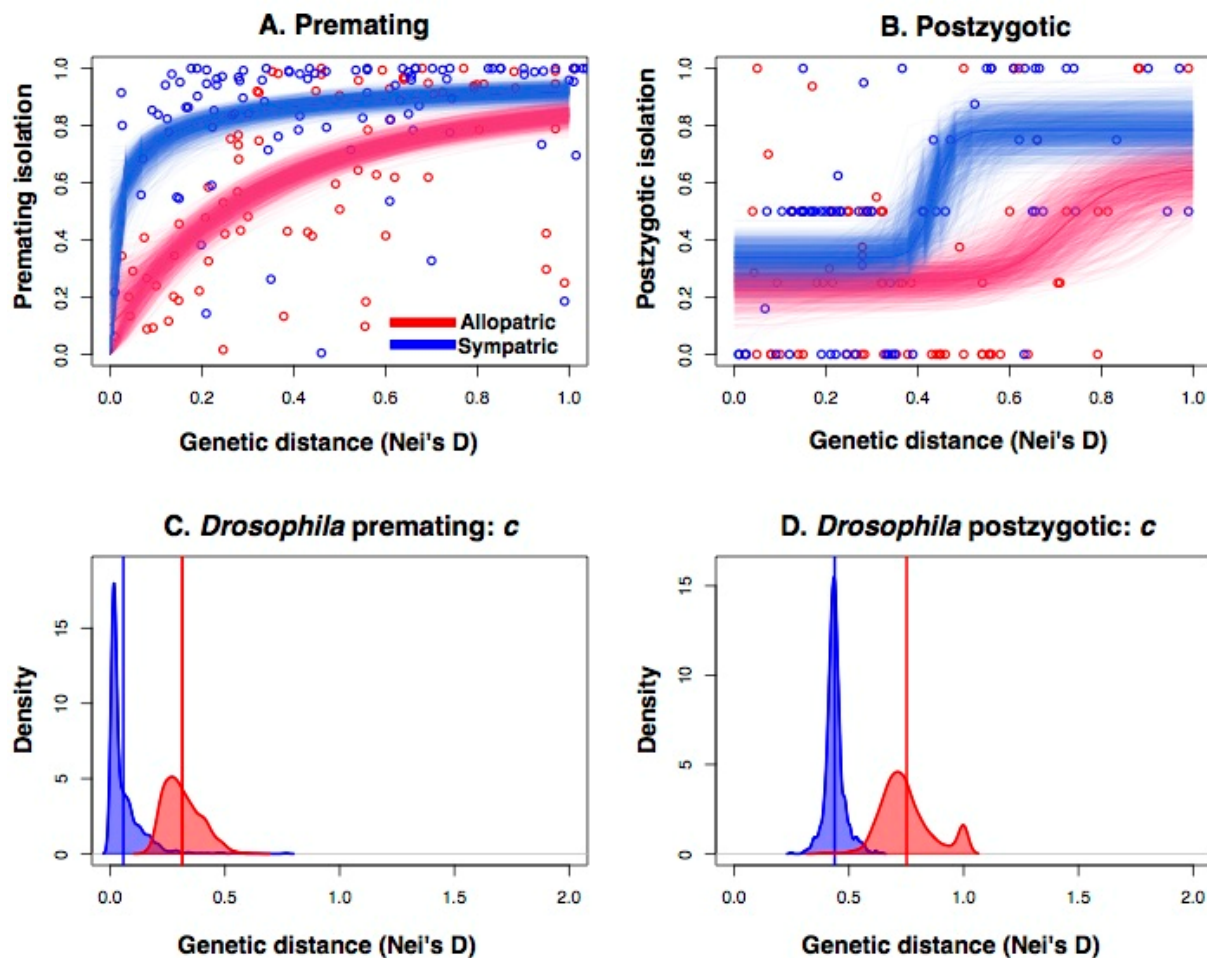
533 We thank J. M. Coughlan and A. Dagilis for comments and J. M. Good for helpful
534 discussions. Research reported in this publication was supported by the National
535 Institute of General Medical Sciences of the National Institutes of Health (NIH) under
536 Award Number R01GM121750 to DRM and R35GM124701 to BSC. The content is
537 solely the responsibility of the authors and does not necessarily represent the official
538 views of the NIH.

539

540 **FIGURES**

541

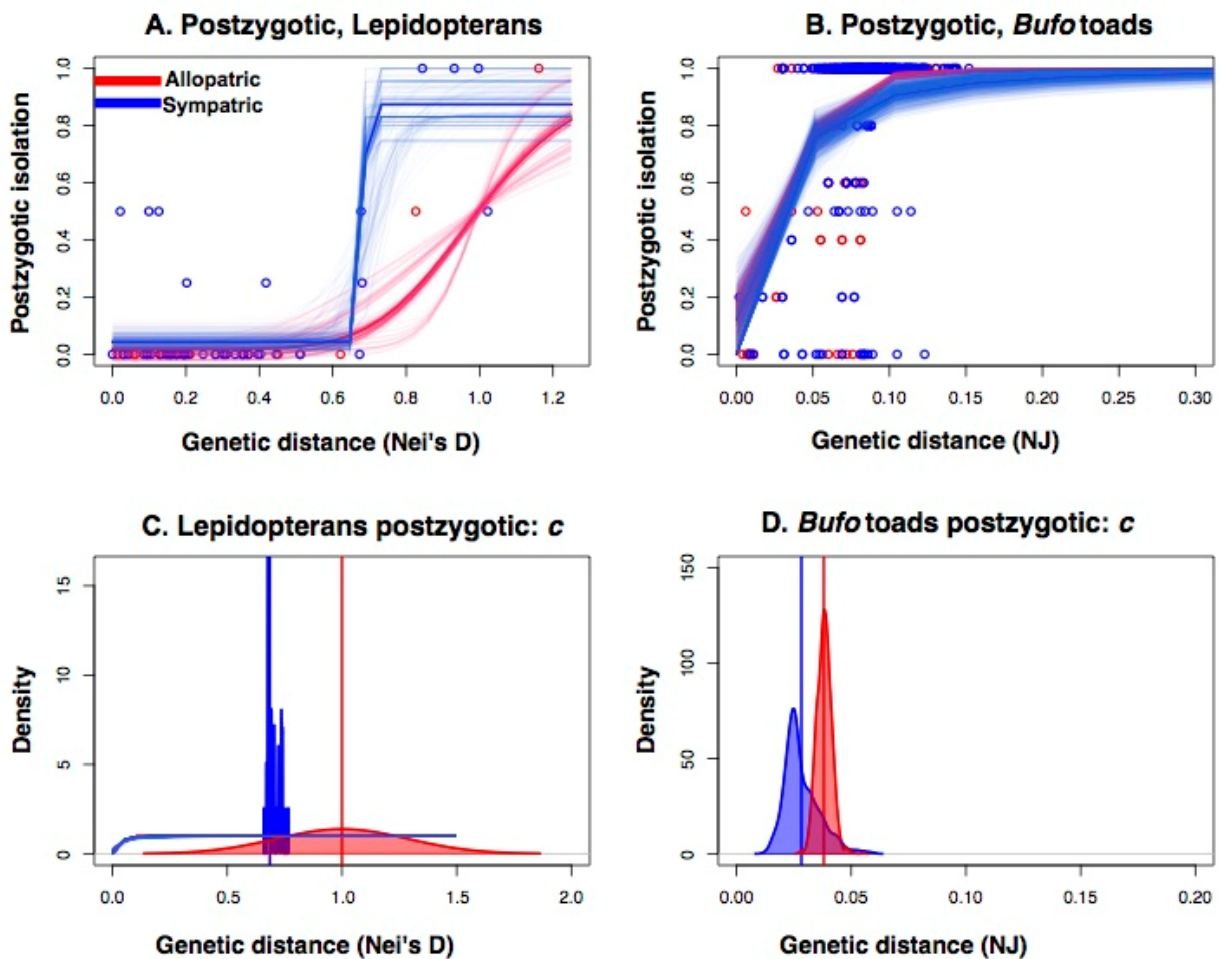
542 **FIGURE 1. Both pre mating and postzygotic RI evolve faster in sympatric than in**
543 **allopatric *Drosophila* species pairs.** Four-parameter logistic models for the evolution
544 of pre mating RI (**A**) and postzygotic RI (**B**) between sympatric (blue) and allopatric (red)
545 *Drosophila* species pairs. Semitransparent lines show 1,000 bootstrapped distributions.
546 For both types of barriers, the inflection point (*c*) is at lower genetic distances for
547 sympatric than for allopatric species pairs (pre mating: **C**; postzygotic: **D**). The vertical
548 lines show the mean value of the bootstrap coefficients. Figure S1 presents the other
549 three parameters for each of the two models.



550

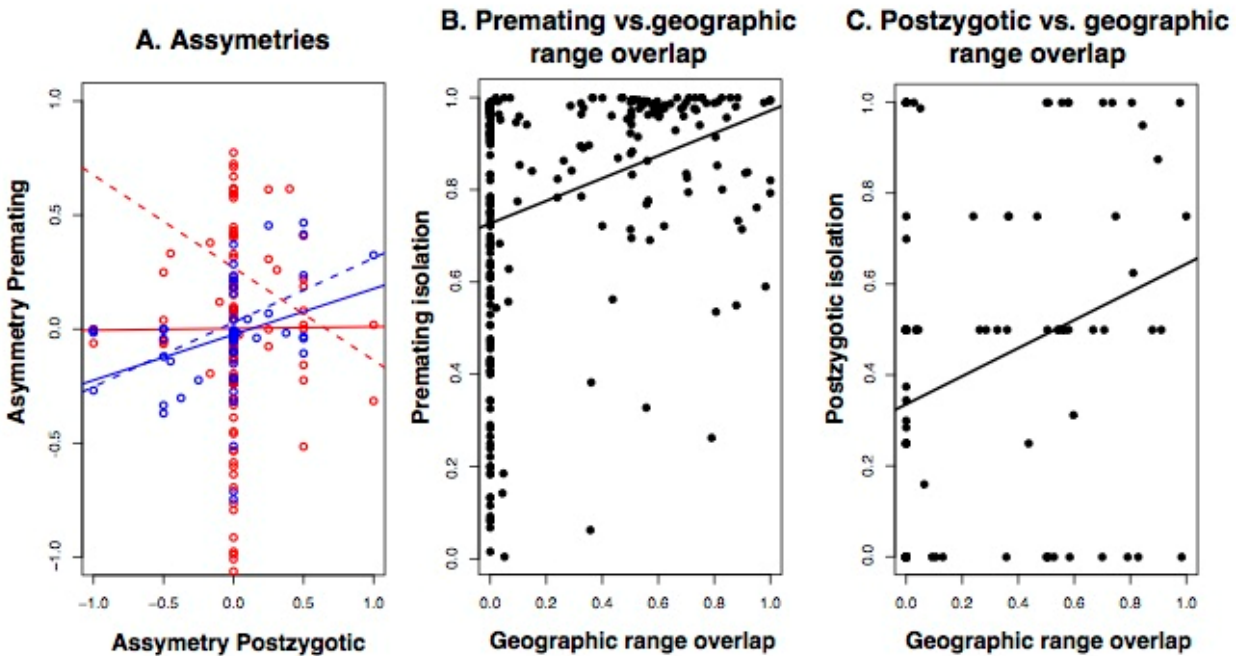
551

552 **FIGURE 2. Postzygotic RI evolves faster in sympatric than in allopatric**
553 **Lepidopteran and *Bufo* toad species pairs.** Four-parameter logistic models for the
554 evolution of postzygotic RI between sympatric (blue) and allopatric (red) Lepidopteran
555 (A) and *Bufo* (B) species pairs. Semitransparent lines show 1,000 bootstrapped
556 distributions. For both barriers, the inflection point (c) is at lower genetic distances for
557 sympatric than for allopatric species pairs (Lepidopterans: C; *Bufo*: D). The vertical lines
558 show the mean value of the bootstrap coefficients. Figure S3 presents the other three
559 parameters for each of the two models. Genetic distance is measured as Nei's D for
560 Lepidopterans and from a neighbor-joining tree for *Bufo* (39).



561
562

563 **FIGURE 3. Proxies of the cost of hybridization are correlated with the strength of**
564 **RI in *Drosophila*. A.** The strength of asymmetries in pre mating RI is weakly correlated
565 with the strength of asymmetries in postzygotic RI in sympatry (solid blue), but not in
566 allopatry (solid red). When completely symmetric pairs (i.e., those where the difference
567 in the strength of RI between reciprocal crosses equals 0) are excluded, asymmetries in
568 pre mating and postzygotic RI in sympatry are no longer significantly correlated (dashed
569 blue line). Range overlap is correlated with the strength of both pre mating (B) and
570 postzygotic RI (C).
571



572
573

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