

1 **Title: Temperature affects conspecific and**
2 **heterospecific mating rates in *Drosophila***

3 **Running Title:** Temperature and mating propensity in *Drosophila*

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17

18 **ABSTRACT**

19 Behavioral mating choices and mating success are important factors in the development
20 of reproductive isolation during speciation. Environmental conditions, especially
21 temperature, can affect these key traits. Environmental conditions can vary across, and
22 frequently delimit species' geographic ranges. Pairing suboptimal conditions with
23 relative rarity of conspecifics at range margins may set the stage for hybridization.
24 Despite the importance of mating behaviors as a reproductive barrier, a general
25 understanding of the interaction between behavioral choices and the environment is
26 lacking, in part because systematic studies are rare. With this report, we begin to bridge
27 that gap by providing evidence that temperature has a significant - but not consistent
28 influence on mating choices and success, and thus on reproductive isolation in
29 *Drosophila*. We studied mating propensity and success at four different temperatures
30 among 14 *Drosophila* species in non-choice conspecific mating trials and in
31 heterospecific trials among two *Drosophila* species triads that are known to regularly
32 hybridize in the wild. We show that mating frequency varies significantly across a 10°C
33 range (from 18°C to 28°C), both in 1:1 mating trials and in high-density *en-masse* trials,
34 but that the effect of temperature is highly species-specific. We also show that mating
35 frequency is consistently low and that temperature has a moderate effect in some
36 heterospecific crosses. As conspecific mating propensity decreases outside of the
37 optimal thermal range, while heterospecific matings remain constant, the proportion of
38 heterospecific matings at suboptimal temperatures is relatively high. This result
39 indicates that temperature can modulate behavioral choices that impose reproductive
40 barriers and influence the rate of hybridization. More broadly, our results demonstrate
41 that to truly understand how mating choice and reproductive isolation occur in nature,
42 they need to be studied in an environmental context.

43 INTRODUCTION

44 Speciation is the process in which one species splits into two lineages which in
45 turn, accumulate genetic differences over time (Coyne and Orr, 2004). These lineages
46 can persist through secondary contact when traits have diverged such that they
47 preclude the formation of hybrids, or when accumulated genetic incompatibilities render
48 hybrids less fit than the parental lineages. Thus, traits that hamper gene exchange
49 between species are crucial for the maintenance of species boundaries. In animals,
50 behavioral mating choices are an important component of speciation, particularly when
51 they result in reproductive isolation (Kirkpatrick and Ravigné, 2002; Mendelson and
52 Safran, 2021; Ritchie, 2007). Comparative studies have revealed that mating
53 discrimination can emerge rapidly between species, and ensues faster than the
54 evolution of postmating-prezygotic isolation (Turissini et al., 2018) and of postzygotic
55 isolation (Coyne and Orr, 1997, 1989; Matute and Cooper, 2021; Turelli et al., 2014).
56 Behavioral isolation has therefore been hypothesized to evolve faster than any other
57 trait involved in reproductive isolation among animals.

58 Barriers to gene flow have often been described as intrinsic or extrinsic
59 depending on whether they are affected by the environmental conditions in which
60 species encounter each other (Coyne and Orr, 2004; Nosil, 2012). Nonetheless, all
61 genetically-based traits, including those that are involved in reproductive isolation, also
62 have an environmental component that interacts with the genome. This means that
63 traits involved in reproductive isolation have interacting genetic and environmental
64 components, and thus the gene \times environment interactions might be a key aspect of
65 species formation and persistence in the face of potential gene flow. Systematic surveys
66 of the strength of different barriers to gene flow in different environments are rare but
67 there is evidence that environmental conditions affect reproductive barriers. For
68 example, immigrants can be less fit (or outright inviable) when they arrive in new
69 environments (Giraud, 2006; Nosil et al., 2005). Additionally, environmental conditions
70 are rarely homogenous across a species' range. Conditions in the core of the range
71 may be more suitable than at its periphery, and theory suggests that this, paired with

72 the relative rarity of conspecifics, may lead to more frequent hybridization at the edge of
73 the geographic range (Wilson and Hedrick, 1982). Similarly, postzygotic isolation
74 barriers, developmentally-based defects that occur after a zygote is formed, have been
75 conventionally considered intrinsic, but systematic studies have shown that
76 environmental conditions can affect the magnitude and impact of these traits. For
77 example, temperature determines the penetrance between negative epistatic
78 interactions that reduce hybrid fitness between *Drosophila* species (Coyne et al., 1998;
79 Miller and Matute, 2017; Presgraves, 2003). Similarly, hybrid newts experience greater
80 oxidative stress at higher temperatures, a phenomenon not observed in the parental
81 species (Petrović et al., 2023). Despite these examples of temperature influencing
82 hybrid inviability, a general understanding about the magnitude of temperature effects in
83 other barriers to gene flow is lacking. A particular aspect that remains understudied in
84 behavioral isolation is how mating behaviors change along continuous environmental
85 gradients, the most important of which might be temperature.

86 Environmental temperature has profound influences on most aspects of life in
87 ectotherms, including numerous aspects of reproduction such as mating and
88 reproductive success (Ingleby et al. 2010, Garcia-Roa et al. 2020, Leith et al. 2021). A
89 recent meta-analysis examined how temperature changes influenced mating latency,
90 choosiness and mating success, three of the components of mating choice in animals
91 (Pilakouta and Baillet, 2022), and suggested an increase in mating success when
92 animals are exposed to higher temperatures during mating trials but not when they were
93 exposed before matings (Pilakouta and Baillet, 2022). This is surprising because for
94 species that exist across an elevational or latitudinal gradient, the concomitant
95 temperature gradient is frequently a key factor that delimits species' geographic ranges.
96 Assessments of the impact of temperature on behavioral isolation are thus important to
97 assess the relative importance of different barriers to interbreeding (Leith et al. 2022).

98 To address this gap, we studied the effect that environmental conditions have on
99 premating isolation in *Drosophila*. In particular, we studied mating rates in both
100 conspecific and heterospecific pairings of 14 *Drosophila* species in different temperature

101 regimes. We used non-choice experiments across a 10°C range (from 18°C to 28°C),
102 observing the responses in both *en-masse* group experiments and 1:1 individual trials.
103 We find that conspecific mate choice is highly contingent on the environmental
104 temperature at which mating takes place, but heterospecific mating rate remains largely
105 constant, and low. Two exceptions occurred among species that are known to hybridize
106 (Comeault et al., 2016; Llopart et al., 2005; Matute and Ayroles, 2014; Schrider et al.,
107 2018), with sexual isolation appearing to be less effective at low temperatures. These
108 results suggest that environmental factors are important in modulating the strength of
109 sexual isolation, with non-optimal environmental conditions perhaps fostering a higher
110 risk of hybridization. Our results further suggest that behavioral choices are similar to
111 other reproductive barriers in their contingency on environmental conditions.

112

113 **MATERIALS AND METHODS**

114

115 **Species and Stocks**

116 Our goal was to determine whether temperature affected mating rate in conspecific and
117 heterospecific crosses of different species of *Drosophila*. We used fourteen *Drosophila*
118 species. Three of the species were from the *yakuba* species complex (*D. yakuba*, *D.*
119 *santomea*, *D. teissieri*), three from the *simulans* species complex (*D. sechellia*, *D.*
120 *simulans*, *D. mauritiana*). We also used *D. melanogaster*, *D. pseudoobscura*, *D.*
121 *willistoni*, *D. paulistorum*, *D. sturtevantii*, *D. virilis*, *D. americana*, and *D. novamexicana*.
122 For all experiments, we used isofemale lines, shelf-stable stocks derived from the
123 progeny from a single female collected in the field (David et al., 2005). Figure 1 shows
124 the phylogenetic relatedness of the species in our sample (based on Kim et al, 2021
125 and Kim et al. 2024), and which species show range overlap in their natural ranges
126 (based on Yukilevich 2012). Table S1 shows the origin, collector, and other details of
127 each isofemale line used in this study. All isofemale lines were maintained in triplicate
128 on corn-meal substrate in 30mL plastic vials since their establishment. Prior to
129 experiments, parallel stocks of flies were reared and maintained in incubators at each of

130 four temperatures (18°C, 22°C, 25°C, and 28°C). We only used one isofemale line per
131 species, and while this limits our ability to assess intraspecific variation, the scale of the
132 experiments did not allow us to include more isofemale lines and remains sufficient to
133 study interspecific effects - the focus of the present work. While previous assessments
134 have detected variation in mating behavior in some of the included species (e.g.,
135 Coughlan et al. 2022, Li et al. 2023, Matute et al. 2010), our goal was to assess the
136 existence of variation in mating behavior, and not quantify the magnitude of the effect of
137 temperature in mating behavior in different genetic backgrounds. The latter goal would
138 require a much larger experiment.

139 Among these 14 species, several species pairs show the ability to produce
140 hybrids. The three species from the *virilis* phylad (*D. virilis*, *D. americana*, and *D.*
141 *novamexicana*) produce fertile offspring in all the reciprocal directions (Hoikkala and
142 Lumme 1987; reviewed in Yukilevich 2012). Genome assessments have also revealed
143 the existence of introgression among these species (Yusuf et al. 2022) but to date, no
144 hybrid zone has been identified for this group. The same is true for the species in the
145 *willistoni* species group (*D. willistoni* and *D. paulistorum*) which show evidence of
146 hybridization (Carson 1954, Dobzhansky and Spassky 1959, Winge and Cordeiro
147 1965), and introgression (Suvorov et al. 2022) but no known stable hybrid zones.
148 Species from the *melanogaster* species group also show evidence of hybridization
149 (reviewed in Turissini et al. 2018) and introgression (Turissini and Matute 2017,
150 Schrider et al. 2018, Suvorov et al. 2022). Three species pairs form stable hybrid zones
151 in the wild: *D. santomea* with *D. yakuba* (Llopart et al. 2004, Matute et al. 2010,
152 Comeault et al. 2016), *D. simulans* with *D. sechellia* (Matute and Ayroles 2014, Schrider
153 et al. 2018) and *D. yakuba* with *D. teissieri* (Cooper et al. 2018). Hybridization is not
154 known from the other species in our sample, despite widespread sympatry (see Figure
155 1). To understand the effects of temperature on interspecific mating behavior, we
156 focused on the *melanogaster* species group, given their occurrence of hybrid zones in
157 nature. We studied the two reciprocal crosses of six hybridizations: *D. santomea* × *D.*

158 *yakuba*, *D. teissieri* × *D. yakuba*, *D. santomea* × *D. teissieri*, *D. simulans* × *D. sechellia*,
159 *D. simulans* × *D. mauritiana*, and *D. sechellia* × *D. mauritiana*.

160

161 **Virgin Collection**

162 About one month before starting experiments, we expanded each isofemale line for the
163 study from 3mL vials into 100mL plastic bottles with cornmeal and yeast (35 g/L, Red
164 Star active dry yeast, *Saccharomyces cerevisiae*). As soon as we observed larvae in
165 the food, we removed the adults, added 1 ml of propionic acid (0.5% v/v) solution to the
166 bottles, and provided a pupation substrate (Kimwipes Delicate Task; Kimberly Clark,
167 Irving, TX). Once we observed black pupae in the KimWipes or the walls of the bottle,
168 we cleared the bottle by transferring all adults to a different bottle. Then, we inspected
169 the bottles every 8 hours and collected any adults that had hatched in that period of
170 time. To separate virgins, we anesthetized all the flies in each bottle on a Flypad with
171 CO₂. Flies were then placed in groups of 20 individuals (separated by sex) in 30mL
172 cornmeal vials.

173

174 **Mating Experiments**

175 We ran two different types of mating experiments: (i) non-choice *en-masse*
176 experiments, and (ii) individual non-choice experiments. We describe the rationale and
177 the procedure for each of them as follows.

178 (i) Non-choice *en-masse*. First, we studied the frequency of conspecific matings (i.e.,
179 females and males from the same species) in *en-masse* experiments. Briefly, we
180 collected females as described immediately above. We combined 100 females and 100
181 males from either conspecific or heterospecific lines in a 30mL vial and housed each
182 female-male combination for 24 hours at each of the 4 temperatures in which they were
183 reared: 18°C, 22°C, 25°C, and 28°C, respectively. For each conspecific, we did five
184 replicates for a total of 280 conspecific *en-masse* matings (14 species × 4 temperature
185 × 5 replicates). After 24h, females were separated using CO₂ anesthesia and the
186 reproductive tract of each female was removed using tweezers. Batches of 5 to 10

187 reproductive tracts were mounted in Ringer's solution (NaCl, KCl, CaCl₂ and NaHCO₃ in
188 distilled water). We inspected both the spermatheca and the seminal receptacle to
189 assess the presence of sperm, which is indicative of mating (Turissini et al., 2018). The
190 proportion of mated females per vial was a proxy of the extent of sexual isolation.

191 To determine whether temperature affected the likelihood of mating in
192 conspecific crosses, we used two different regression families. First, we fit a binomial
193 logistic regression in which female mating status was the response, species was a fixed
194 effect, and temperature was a continuous effect. The model also included the
195 interaction between sex and temperature. We used the R function *glm* (family =
196 "binomial" (library *stats*, R Core Team, 2018) to fit the models. To determine the
197 significance of each effect, we used likelihood ratio tests using the function *lrtest* (library
198 *lmerTest*, Hothorn et al., 2015) in which we compared a model with and without the effect
199 to be tested. The logistic linear model followed the form:

200

201 $\text{mating_status} \sim \text{cross} + \text{temperature} + (\text{cross} \times \text{temperature})$ [Equation 1]

202

203 We also fit a logistic regression that included a quadratic temperature term and
204 the interaction with cross. The model followed the form:

205

206 $\text{mating_status} \sim \text{cross} + \text{temperature} + \text{temperature}^2 + (\text{temperature} \times \text{cross}) + (\text{cross} \times$
207 $\text{temperature}^2)$ [Equation 2]

208

209 Second, we studied the frequency of heterospecific matings (i.e., females and
210 males from different species), also in an *en-masse* setting. Table S2 lists the possible
211 interspecific crosses included in this portion of the research. For each heterospecific
212 mating, we did five replicates for a total of 60 interspecific assays (6 crosses × 2
213 directions × 5 replicates). We scored the proportion of mated females in the same way
214 as described for conspecific matings (immediately above).

215 For each type of interspecific cross, we fit a binomial regression in which the
216 mating status of the dissected female was the response, the direction of the cross was a
217 fixed effect, and temperature was a continuous effect. The model also included the
218 direction \times temperature interaction. The linear logistic model had the following form:

219
220
$$\text{mating_status} \sim \text{direction} + \text{temperature} + (\text{direction} \times \text{temperature})$$
 [Equation 3]

221
222 We also fitted a second model including the quadratic temperature term which
223 took the form:

224
225
$$\text{mating_status} \sim \text{direction} + \text{temperature} + \text{temperature}^2 + (\text{temperature} \times \text{direction}) +$$

226 $(\text{direction} \times \text{temperature}^2)$ [Equation 4]

227
228 In total, we fit twelve regression models for these analyses (six linear and six quadratic).
229 We compared the model fit using AIC values (calculated using the function `AIC`, library
230 `stats`, R Core Team 2018) and only retain the quadratic model if $\text{AIC}_{\text{Quadratic}}$ had a better
231 value than the $\text{AIC}_{\text{Linear}}$ by two AIC units. Every model described here (conspecific or
232 heterospecific), was followed by posthoc Tukey HSD tests using the function `glht`
233 (library `multcomp`, (Hothorn et al., 2016, 2008)).

234
235 (ii) Non-choice individual pairs. We also conducted experiments in which we watched
236 pairs of flies (one male and one female) to study two key characteristics of sexual
237 behavior, namely copulation latency (the time that it takes for copulation to begin) and
238 copulation duration (the time that coupling lasts). We measured copulation latency and
239 duration in both conspecific and heterospecific crosses using non-choice mating
240 experiments. Matings all were run in a climate-controlled room at four different
241 temperatures (18°C, 22°C, 25°C, and 28°C) corresponding with the temperatures at
242 which the flies were reared. All flies in this experiment were collected as virgins and
243 housed in single sex vials as described in the section immediately above (Virgin

244 collection). On day four after hatching, one female and one male were aspirated into a
245 single, empty vial. All mating trials were started within 1 hr of the beginning of the light
246 cycle to maximize fly activity and female receptivity. We observed 100 pairs per
247 genotype pairing, and the flies were watched constantly for 1 hour. No more than 200
248 vials were set up in parallel to ensure accuracy in recording when copulation began and
249 ended. For each of the pairs, we recorded whether mating took place, and in cases
250 where copulation occurred, we timed latency (the time to copulation initiation) and
251 duration (time from mounting to separation).

252 To determine whether temperature had an effect on mating frequency in
253 conspecific matings, we fit a linear and quadratic logistic models identical to the ones
254 we used for *en-masse* experiments (Equations 1 and 2). We also use AIC values to
255 determine which model was a better fit to the data.

256 We compared the results of the individual trials to those of the *en-masse*
257 experiments using a set of Pearson's correlation tests (function *corr*, library *stats*, R
258 Core Team 2018) of mating rate in *en-masse* trials vs. mating rate in individual trials at
259 each of the four test temperatures. We also fit quadratic models to study the effect of
260 temperature in conspecific mating latency and duration. The models were identical to
261 those presented in equations 1 and 2, except that they were not logistic but gaussian
262 models.

263 We followed a similar approach to analyze data from heterospecific matings. For
264 each hybridization in which we obtained latency and duration data from both directions
265 of the cross (five hybridizations), we fit a linear model for each of the mating traits with
266 the direction of the cross as a fixed effect, and temperature as a continuous effect, and
267 included an interaction between these two effects (i.e., a model similar to equation 3 but
268 with a gaussian distribution instead of a logistic one). Similarly, we conducted model fit
269 assessments using the models' AIC values. The cross *D. sechellia* × *D. mauritiana*
270 yielded no matings during the 1-hour observation period at any of the temperatures,
271 thus we did not conduct statistical analyses for this hybridization.

272 Finally, we used the rates of mating in conspecific matings and heterospecific
273 matings to calculate an index of sexual isolation for each direction of interspecific
274 crosses. The index, I_S , normalizes the proportion of heterospecific matings in a direction
275 by the number of matings in conspecific crosses involving the same female genotype.
276 The index follows the form:

277

$$278 \quad I_S = 1 - \left(\frac{\text{proportion of heterospecific matings}}{\text{proportion of conspecific matings}} \right)$$

279

280 I_S usually ranges between 0 (when heterospecific matings are as common as
281 conspecific matings) and 1 (when heterospecific matings do not occur), however
282 negative values can occur and indicate that heterospecific matings are more common
283 than the conspecifics. We used the Agresti-Coull Interval Method to calculate the
284 confidence intervals for the heterospecific and conspecific mating proportions (function
285 *add4ci*, library *PropCIs*, Scherer and Scherer, 2018) and a 2-sample test for equality of
286 proportions with continuity correction (function *prop.test*, library *stats*, R Core Team,
287 2018) to compare mating proportions.

288

289 **Ethical Note**

290 The species in this study do not require licenses or permits. Mated females were
291 dissected under CO₂ anesthesia to minimize pain. All flies were killed by immersion in
292 isopropanol after experimentation.

293 **RESULTS**

294

295 Temperature effect in conspecific and heterospecific *en-masse* matings.

296 First, we studied whether environmental temperatures affected the likelihood of mating
297 between conspecifics in *en-masse* matings. Figure 2A shows the relative frequency of

298 mating for 14 species at four temperatures. A logistic regression with binomial
299 responses on the *en-masse* experiments conducted at different temperatures revealed
300 that environmental temperature had a strong effect on whether mating took place, but
301 also strong heterogeneity among species suggests differential effects of temperature
302 across species. Notably, we find a strong interaction between temperature and species
303 identity suggesting that the effect of temperature varies among species and that its
304 magnitude is not universal (Table 1). Adding a quadratic temperature effect also
305 showed strong effects of species, temperature, and the interaction between these two
306 effects, but also revealed a significant contribution of the squared temperature, and the
307 interaction between the squared temperature and species (Table 1). Notably, the
308 addition of the quadratic term improves the model fit ($AIC_{\text{Linear}} = 36,412.97$; $AIC_{\text{Quadratic}} =$
309 $33,057.5$), indicating that the likelihood of conspecific mating is better explained by a
310 nonlinear model. Figure S1 shows the proportion of mated females for each species
311 along the temperature continuum.

312 Next, we studied whether temperature had an effect on the frequency of
313 heterospecific matings in the *en-masse* setting. For these analyses, we also used linear
314 and quadratic and logistic regressions. In five of the hybridizations, the quadratic model
315 fit better than the linear model (AIC values listed in Table S3). The only exception was
316 the *D. mauritiana* × *D. sechellia* crosses in which the linear model was a better fit. In
317 four of the hybridizations (*D. yakuba* × *D. santomea*, *D. yakuba* × *D. teissieri*, *D.*
318 *santomea* × *D. teissieri*, and *D. simulans* × *D. mauritiana*), either the temperature effect
319 or the square temperature effect were significant, indicating that the likelihood of
320 heterospecific mating in two directions of the cross was affected by temperature (Table
321 2 and Table S4). The same four hybridizations showed a significant interaction between
322 the square of the temperature and the direction of the cross. Two crosses also showed
323 a significant interaction between temperature and cross direction. The significance of
324 these interactions indicates a differential effect of temperature in the two directions of
325 the cross in these hybridizations. Notably, these interactions are significant in the three

326 hybridizations of the *yakuba* species group, a group of species with marked interspecific
327 differences in thermal fitness (Comeault and Matute, 2021; Cooper et al., 2018; Matute
328 et al., 2009). Table S4 shows the results for the linear models, which also show a
329 species-dependent effect of temperature, but suggest a much stronger effect of the
330 reciprocal cross direction. The crosses *D. mauritiana* × *D. sechellia* showed no strong
331 effect of temperature in the linear or quadratic analyses, but do note that the number of
332 matings in these two reciprocal crosses was low.

333

334 Temperature effect in individual non-choice matings.

335 *En-masse* mating experiments allow rapid quantification of the rate of
336 insemination in a combination of genotypes but make the measurement of individual
337 mating components a challenge. We thus set up individual non-choice experiments (i.e.,
338 one male and one female). First, we studied whether temperature had an effect on
339 mating success, copulation latency, and copulation duration in individual conspecific
340 non-choice experiments. Figure 3 shows the mating frequency, latency, and duration in
341 conspecific matings across 14 *Drosophila* species. Similar to our results in the *en-*
342 *masse* experiments, both linear and quadratic models revealed that the likelihood of
343 mating is affected by temperature, species identity, and the interaction between
344 temperature and species. For mating frequency, the quadratic model shows a better fit
345 than the linear model ($AIC_{\text{Quadratic}} = 6,555.599$, $AIC_{\text{Linear}} = 7,136.356$), and revealed a
346 non-linear effect of temperature and a strong interaction between the quadratic term
347 and species identity (Table 3). These results are similar to those of the *en-masse*
348 experiments, which is not surprising given that the mating frequency in these individual
349 non-choice experiments was highly correlated with the mating frequency in the non-
350 choice *en-masse* experiments described immediately above (Figure 4, Table S5). Table
351 S6 shows linear and quadratic regressions for the mating frequency of each species

352 independently, which indicates that temperature was a significant effect in the best
353 fitting model for 12 of 14 species.

354 To quantify the individual components of conspecific mating behavior, we fit
355 linear and quadratic models to identify sources of heterogeneity in mating latency and
356 mating duration in non-choice individual conspecific crosses. The addition of a quadratic
357 term did not improve the model fit for conspecific copulation latency ($AIC_{\text{Quadratic}} =$
358 $17,089.44$, $AIC_{\text{Linear}} = 17,088.44$). Mating latency, or the time that it takes for mating to
359 start, did not differ across species (Species effect: $F_{13,2163} = 1.2972$, $P = 0.2067$), and
360 was not influenced by temperature (Temperature effect: $F_{1,2163} = 2.2835$, $P = 0.1309$,
361 Figure 3B). There was no species-specific effect of temperature either (temperature \times
362 sex interaction: $F_{1,2163} = 1.403$, $P = 0.150$). These results indicate no generalized
363 across-species effect of temperature on conspecific mating latency. Table S7 shows
364 linear and quadratic regressions for the mating latency of each conspecific cross
365 independently which indicates that temperature has some effect in three species, *D.*
366 *yakuba*, *D. teissieri*, *D. melanogaster*, and *D. willistoni*. Figures S2 and S3 shows the
367 mating latency for each species along the temperature continuum.

368 Mating duration in conspecific crosses, unlike latency, was better explained by a
369 quadratic model than by a linear one ($AIC_{\text{Quadratic}} = 17,979.11$; $AIC_{\text{Linear}} = 18,045.79$).
370 Duration was affected by species identity, temperature, the quadratic form of
371 temperature, and the two interactions between cross and temperature (Table 4). These
372 results suggest that while temperature had no detectable effect on mating latency in
373 conspecific crosses, mating duration was influenced by temperature in a species-
374 specific manner. Table S8 shows the fit for linear and quadratic regressions for each of
375 the 14 species. Temperature influenced mating duration in all the best-fitting
376 regressions (Table S8), suggesting that conspecific mating duration was affected by
377 temperature in all species. Figures S4 and S5 shows the mating duration for each
378 species along the temperature continuum.

379 Next, we studied the effect of temperature on the likelihood of mating in non-
380 choice individual heterospecific assays. The quadratic models showed a better fit than
381 linear models in two species pairs (*D. yakuba* × *D. santomea* and *D. simulans* × *D.*
382 *mauritiana*, Table S9). Neither linear logistic nor quadratic logistic models show a
383 generalized effect of temperature (Table 5 and Table S10). Unlike our analyses using
384 *en-masse* matings, we found no significant interaction between cross direction and
385 temperature in any of the hybridizations (Table 5) indicating differences in results
386 depending on the experimental approach to study mating behavior in heterospecific
387 crosses. Note that these experiments had fewer observations than *en-masse*
388 experiments and that we observed no copulations for the ♀ *D. sechellia* × ♂ *D.*
389 *mauritiana* cross at any temperature in this assay.

390 From these individual heterospecific non-choice experiments, we obtained
391 latency and duration measurements for eleven of the twelve interspecific crosses, or
392 five bidirectional hybridizations and one unidirectional cross. In the five cases for which
393 we had data for the two reciprocal crosses, the inclusion of quadratic terms did not
394 improve the models fit to the latency or duration data. Tables S11 and S12 show the
395 AIC values for the linear and quadratic models for heterospecific mating latency and
396 duration, respectively. Linear models fit better for both latency and duration in all
397 hybridizations. Mating latency in crosses between *D. simulans* and *D. sechellia* was the
398 only instance in which the direction of the cross, temperature and the interaction
399 between these two effects were significant (Table 6). These results indicate that
400 temperature had no across-species effect on mating latency or duration in
401 heterospecific crosses in *Drosophila* in timed individual mating trials.

402 Finally, we calculated an index of sexual isolation (I_s) as a proxy of the strength
403 of reproductive isolation resulting from mating choice at each temperature. This index
404 represents the proportional risk of hybridization when normalizing by the number of
405 conspecific matings that occur at any given temperature. Figure 6 shows the effect of
406 temperature on isolation for *en-masse* and individual mating experiments. Sexual

407 isolation is complete, or almost complete, in some crosses (i.e., ♀*D. sechellia* × ♂*D.*
408 *mauritiana* and ♀*D. yakuba* × ♂*D. teissieri*), regardless of the temperature. On the other
409 hand, the crosses between ♀*D. yakuba* × ♂*D. santomea* and between ♀*D. simulans* ×
410 ♂*D. sechellia* (and to a lesser extent ♀*D. santomea* × ♂*D. yakuba*) showed stark
411 differences in total sexual isolation across temperatures with heterospecific matings
412 being as common as conspecific at 18 and 28°C, the extreme values of the assayed
413 temperature range. Despite finding some mean I_S values < 0, we did not find any
414 significant instances where the proportion of heterospecific matings exceeded the
415 proportion of conspecific matings ($\chi^2 < 2.602$, $df = 1$, $P > 0.1067$). These results indicate
416 that the magnitude of sexual isolation as a barrier to gene flow might be contingent
417 upon temperature, at least in some interspecific crosses.

418 **DISCUSSION**

419 In this report, we describe the effect of temperature on conspecific-mating frequency
420 and behavior in fourteen species of *Drosophila*, and find that while temperature is a
421 determinant of the likelihood of mating, the effect is highly species-specific. We also find
422 that the effect of temperature on the frequency of heterospecific matings in six
423 interspecific hybridizations is moderate. These results have three implications. First,
424 they indicate that mating propensity is dependent upon environmental temperature in
425 *Drosophila*. Since flies use external temperature to thermoregulate, it follows that flies
426 should be more receptive in their optimal temperature range, and that they will be less
427 receptive at stressful temperatures. Second, our results suggest that environmental
428 temperature might influence hybridization rates between species not by increasing the
429 likelihood of heterospecific matings, but rather by reducing the likelihood of conspecific
430 matings. Collectively, these results indicate that conditions in which mating takes place
431 are an overlooked component of the experimental design of behavioral mating
432 experiments in animals.

433 Our study follows in the steps of previous research that has suggested that while
434 temperature can affect mating behaviors, that effect is variable. A meta-analysis on the
435 effect of within-species mating behavior across animals suggested no consistent
436 directional effect of temperature on mating behaviors and mating success (Pilakouta
437 and Baillet, 2022). These observations are consistent with our results and both studies
438 highlight the difficulties of predicting changes in the strength of sexual selection among
439 natural populations in a warming world (Pilakouta and Ålund 2021). One potential
440 mechanism for the differences in mating likelihood is that individuals are more
441 physiologically stressed in maintaining homeostasis at higher temperatures, and that
442 they are simply not active at lower temperatures. Other possibilities also exist.
443 Temperature might affect mating by modulating the transduction or reception of
444 courtship signals. Rearing temperature affects cuticular hydrocarbon profiles, which can
445 be important in mate choice across insects (Conrad et al., 2017; Duarte et al., 2019;
446 Kárpáti et al., 2023; Michelutti et al., 2018; Noorman and Otter, 2002; Rajpurohit et al.,
447 2021; Savarit and Ferveur, 2002; Silva et al. 2007; Westerman and Monteiro 2016).
448 Mating temperature affects visual and vibratory courtship behaviors in spiders,
449 (*Habronattus clypeatus*, Brandt et al., 2020, 2018; *Schizocosa floridana*, Rosenthal and
450 Elias, 2019). In rock lizards (*Iberolacerta cyreni*), chemosensory signals are less likely
451 to be effectively conducted at high temperatures (Martín and López, 2013). Dissecting
452 the precise molecular, physiological, and neurological underpinnings of the interaction
453 between mating choice and temperature will be of critical importance to understand
454 species boundaries in the face of global warming (Chunco, 2014; Groot and Zizzari,
455 2019; Muhlfeld et al., 2014; Vallejo-Marín and Hiscock, 2016).

456 The second implication of our findings is the effect of temperature on the
457 likelihood of heterospecific matings. Our findings indicate that while the rate of
458 conspecific matings is highly contingent on environmental temperature, the rate of
459 interspecific matings is roughly similar across temperatures. These results imply that
460 environmental conditions can play a key role in the proportional representation of

461 heterospecific vs. conspecific matings, and therefore in the establishment and
462 persistence of hybrid zones. It has been suggested that instances of hybridization might
463 be more likely to occur at the edge of a geographic range because of the relative rarity
464 of conspecifics (and abundance of heterospecifics, Wilson and Hedrick, 1982), but
465 hybrid zones also tend to occur at the edge of species limits where physiological
466 boundaries are pushed. In the case of *Drosophila*, *D. santomea* and *D. yakuba*
467 hybridize in the midlands of the oceanic volcano of São Tomé (Lachaise et al., 2000;
468 Llopart et al., 2005; Matute, 2010) where the hybrid zone occurs at the upper edge of
469 the thermal range for *D. santomea*, and at the lower edge for *D. yakuba*. The
470 competitive outcomes between these two species are mediated by temperature, with *D.*
471 *yakuba* outperforming *D. santomea* in warmer conditions (Comeault and Matute, 2021).
472 Similarly, *D. teissieri* and *D. yakuba* hybridize in the highlands of another island, Bioko,
473 at the lower end of the thermal range of *D. teissieri* (Cooper et al., 2018). Our
474 experiments here reflect the importance of thermal fitness differences in these species
475 pairs and demonstrate that the likelihood of mating in both *D. santomea* and *D. teissieri*
476 —the thermally sensitive species—is affected more strongly by temperature than that of
477 *D. yakuba*.

478 Other environmental conditions, besides temperature, are also important
479 modulators of mating behavior and reproductive isolation. Temperature, density, and
480 age are all environmental factors that affect the extent of phenotypic variation in traits
481 that lead to behavioral isolation between nascent and well-formed species, alike. Both
482 modeling (Reeve, 1989; Shizuka and Hudson, 2020; Wilson and Hedrick, 1982) and
483 experimental studies (Friberg et al., 2013; Gomez-Llano et al., 2018; Keränen et al.,
484 2013; Matute, 2014) have shown that the density of heterospecifics is an important
485 factor in mating propensity. Speciation can be impeded by mating choice, as individuals
486 that have few choices might pursue matings with heterospecifics, even if the offspring
487 are somehow less fit, depending on the waiting time for a potential conspecific mate
488 (Chen and Pfennig, 2020; Wilson and Hedrick, 1982). This may be especially

489 pronounced in species that have low abundance at the edge of their range, and which
490 have the potential to hybridize at that interface. A third component that might determine
491 the likelihood of heterospecific matings is the age of the individuals engaging in the
492 cross. In *Aedes* mosquitos, older individuals tend to engage in heterospecific matings
493 more readily than younger ones (Bargielowski et al., 2019). The density of conspecifics
494 and heterospecifics, their age, and environmental temperature might all covary and their
495 relative effect in reproductive isolation might be challenging to dissect in field conditions.

496 Besides sexual isolation, developmentally-based reproductive isolation is also
497 affected by environmental conditions, and genetic studies provide a mechanism for this
498 genetic x environment interaction. A handful of studies have shown that hybrid inviability
499 and hybrid sterility are both dependent on temperature (Coyne et al., 1998; Lee 1978;
500 Mason et al., 2011; Presgraves, 2003; Wongpatsa et al., 2014). Hybrids between
501 *Drosophila* species show higher levels of inviability at higher temperatures (Barbash et
502 al., 2000; Hutter and Ashburner, 1987; Lee, 1978; Matute et al., 2010; Sawamura et al.,
503 1993), and genome-wide mapping suggests that this effect is generalized across the
504 genome. In *melanogaster/santomea* and *melanogaster/simulans* hybrids, the
505 penetrance of different genomic regions involved in inviability is greater at higher
506 temperatures than at low temperatures (Coyne et al., 1998; Matute et al., 2010; Miller
507 and Matute, 2017; Presgraves, 2003). Several genetic loci involved in hybrid fitness
508 reductions at 24°C have diminished effects at lower temperatures. In hybrids between
509 *Tribolium* beetle species, hybrid male viability declines as temperature increases,
510 leading to increasing manifestation of Haldane's rule at high temperatures (Wade et al.,
511 1999). These previous results suggest that reproductive isolation even between long-
512 diverged species is conditioned by temperature. The effect of age is not limited to
513 premating isolation and can also affect the strength of postzygotic isolation. In hybrids
514 between the subspecies of *D. pseudoobscura*, hybrid males are weakly fertile but only
515 when they are old (Orr and Irving, 2005). These instances suggest that even for barriers

516 that are often thought to be intrinsic, ecological factors might be of critical importance
517 (Anderson et al., 2023).

518 Our study also has some important caveats. The flies in our study (both male and
519 female) were kept in temperature-controlled conditions throughout the trials and they
520 were reared in the same temperature regimes at which they were tested. We are
521 therefore unable to comment on any influence on mating preferences among these
522 species that might arise from development in other temperature conditions or in
523 fluctuating conditions. Reproductive traits like fecundity and fertility are also dependent
524 on developmental temperature in *Drosophila* (Huey et al. 1995; Nuney and Cheung
525 1997; Matute et al. 2009; Kelpsattel et al. 2019; Comeault et al. 2020) and other insects
526 (Kersting et al. 1999, Papanikolaou et al. 2013, Cui et al. 2018, Li et al. 2020).
527 Understanding how thermal regimes during development interact with climate exposure
528 as reproductive adults work to shape mating choices thus merits follow-up. Second, our
529 experiments are necessarily unrealistic. *En-masse* experiments tested female choice
530 when presented with multiple males simultaneously generating male-male, and female-
531 female interactions, which our experimental design does not allow us to quantify.
532 Similarly, individual non-choice experiments include only one female, and one male,
533 which might represent an oversimplification of natural matings. It remains possible that
534 females might react differently when presented with males in a more sequential fashion,
535 and that the order in which a female encounters con-vs. heterospecific males might alter
536 her mating preferences or other aspects of her physiology (e.g., Matute and Coyne
537 2010).

538 Our report serves as a survey demonstrating that behavioral traits are affected by
539 environmental conditions. While this is not a particularly surprising result, interactions
540 between environmental factors and the alleles that underlie behavior and reproductive
541 isolation remain largely understudied. In order to truly understand how mating choice
542 and reproductive isolation occur in nature, they need to be studied in the context of the
543 conditions in which they take place.

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804 **TABLES**

805

806 **TABLE 1. Temperature affects the likelihood of conspecific mating in enmasse settings.**

807 We show the model fit for the linear and quadratic models.

	Linear			Quadratic		
	LRT	df	P	LRT	df	P
Species	13.472	1	2.421×10^{-4}	1,467.21	13	$< 1 \times 10^{-10}$
Temperature	1,320.749	1	$< 1 \times 10^{-10}$	2,828.02	1	$< 1 \times 10^{-10}$
Temperature ²	NA	NA	NA	2,859.67	1	$< 1 \times 10^{-10}$
Species x temperature	613.909	13	$< 1 \times 10^{-10}$	521.82	13	$< 1 \times 10^{-10}$
Species × temperature ²	NA	NA	NA	523.80	13	$< 1 \times 10^{-10}$

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810

811 **TABLE 2. Quadratic logistics regressions suggest a common effect of temperature in the**
 812 **likelihood of heterospecific mating in *en-masse* experiments.** The metric of isolation is
 813 receptivity of females in *en-masse* matings. All the likelihood ratio tests (LRT) comparisons
 814 involve one degree of freedom.

Species pair	Direction		Temperature		Direction x temperature		Temperature ²		Temperature ² x direction	
	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
<i>D. yakuba</i> / <i>D. santomea</i>	7.238	0.007	10.527	1.177 × 10⁻³	7.836	5.122 × 10⁻³	11.532	6.841 × 10⁻⁴	6.360	0.012
<i>D. yakuba</i> / <i>D. teissieri</i>	0.003	0.958	24.299	8.249 × 10⁻⁷	0.046	0.830	25.419	4.613 × 10⁻⁷	<u>0.067</u>	<u>0.796</u>
<i>D. santomea</i> / <i>D. teissieri</i>	0.003	0.955	12.267	4.612 × 10⁻⁴	0.014	0.905	14.509	1.395 × 10⁻⁴	<u>0.014</u>	<u>0.907</u>
<i>D. simulans</i> / <i>D. sechellia</i>	1.682	0.195	1.342	0.247	3.739	0.0532	1.764	<u>0.184</u>	4.564	0.0327
<i>D. simulans</i> / <i>D. mauritiana</i>	7.060	0.008	18.214	1.975 × 10⁵	6.091	0.014	18.799	1.452 × 10⁻⁵	6.763	9.309 × 10⁻³
<i>D. sechellia</i> / <i>D. mauritiana</i>	3.190 × 10 ⁻⁴	0.986	0.033	0.855	1.058 × 10 ⁻³	0.974	0.095	<u>0.755</u>	7.973 × 10⁻³	<u>0.929</u>

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816

817 **TABLE 3. Temperature affects the likelihood of conspecific mating in individual settings.**

818 We show the model fit for the linear and quadratic models. The significance of each effect was

819 determine with Likelihood ratio tests.

	Linear			Quadratic		
	LRT χ^2	df	P	LRT χ^2	df	P
Temperature	40.809	1	1.679×10^{-10}	464.29	1	$< 1 \times 10^{-10}$
Species identity	95.939	13	$< 1 \times 10^{-10}$	296.42	13	$< 1 \times 10^{-10}$
Temperature x species	136.218	13	$< 1 \times 10^{-10}$	134.18	13	$< 1 \times 10^{-10}$
Temperature ²	NA	NA	NA	473.21	1	$< 1 \times 10^{-10}$
Species x Temperature ²	NA	NA	NA	135.55	13	$< 1 \times 10^{-10}$

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823 **TABLE 4.** The identity of the cross and temperature have an effect on copulation duration of
824 conspecific crosses.

Effect	df	F	P
temperature ²	1	24.410	8.386 × 10 ⁻⁷
temperature	1	26.315	3.161 × 10 ⁻⁷
cross	13	3.274	5.950 × 10 ⁻⁵
temperature ² x cross	13	3.066	0.0001606
Temperature x cross	13	3.241	6.965 × 10 ⁻⁵

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826

827 **TABLE 5. Temperature has a moderate effect on mating propensity in some of the six**
 828 **types of heterospecific *individual* matings.** The metric of isolation is receptivity of females in
 829 *en-masse* matings. All the likelihood ratio tests (LRT) comparisons involve one degree of
 830 freedom. Quadratic logistics.

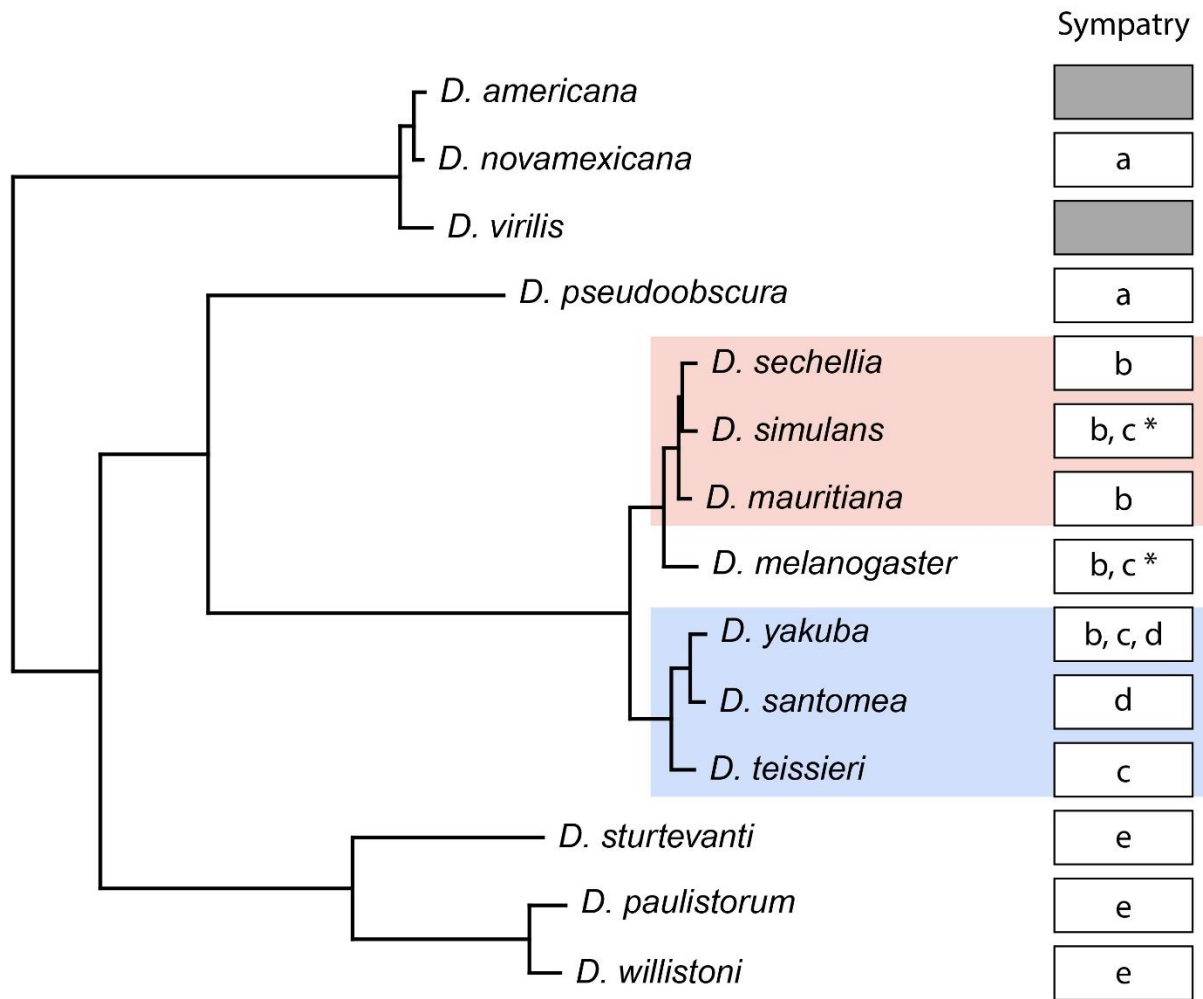
Species pair	Direction		Temperature		Temperature ²		Direction x temperature		Direction x temperature ²	
	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P
<i>D. yakuba</i> / <i>D. santomea</i>	2.141	0.143	1.645	0.200	1.828	0.176	2.366	0.124	0.059	0.151
<i>D. yakuba</i> / <i>D. teissieri</i>	0.512	0.474	5.054	0.025	5.198	0.023	0.575	0.448	0.578	0.447
<i>D. santomea</i> / <i>D. teissieri</i>	0.012	0.913	0.634	0.426	0.848	0.357	0.006	0.939	0.007	0.934
<i>D. simulans</i> / <i>D. sechellia</i>	0.620	0.431	0.123	0.726	0.138	0.711	1.018	0.313	1.100	0.294
<i>D. simulans</i> / <i>D. mauritiana</i>	2.277	0.131	8.104	0.004	7.652	0.006	1.797	0.1801	1.576	0.209
<i>D. sechellia</i> / <i>D. mauritiana</i>	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

832 **FIGURES**

833 **FIGURE 1. Phylogeny and sympatry of *Drosophila* species included in this study.**

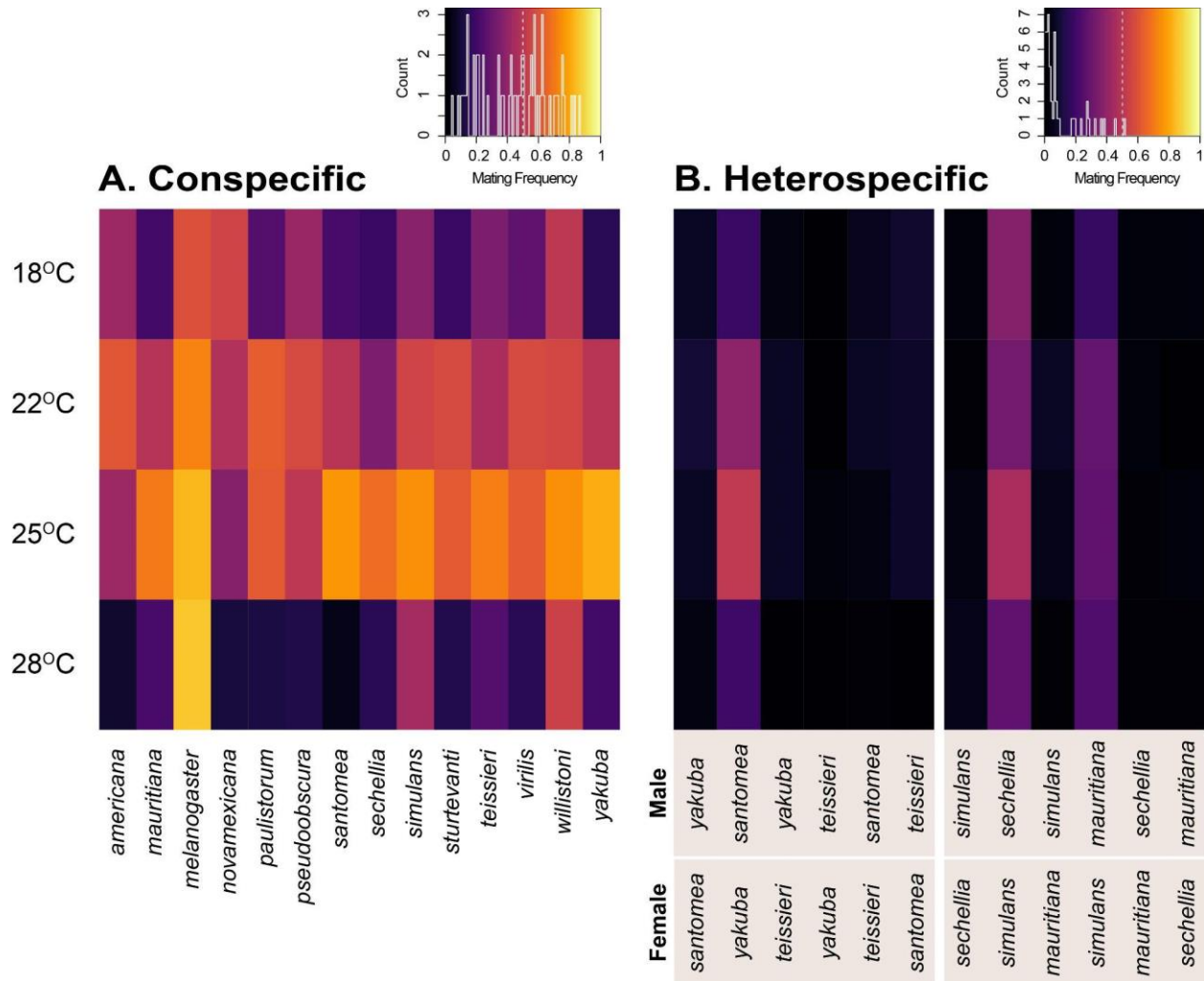
834 The phylogeny here was pruned from the Kim et al. (2021, 2024) phylogeny to include
835 the 14 species in our mating choice trials. These include two recently-diverged species
836 triads with species that are known to hybridize in the wild (red and blue shaded
837 regions). Boxes show which species share overlapping geographic ranges.

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840

841 **FIGURE 2. Mating frequency in *en-masse* mating experiments. A.** Mating frequency
 842 in conspecific pairings of 14 different species of *Drosophila*, measured by counting
 843 inseminated females from groups of 100 females and 100 males. **B.** Mating frequency
 844 in reciprocal heterospecific matings between six *Drosophila* species pairs, similarly
 845 measured as inseminated females.
 846



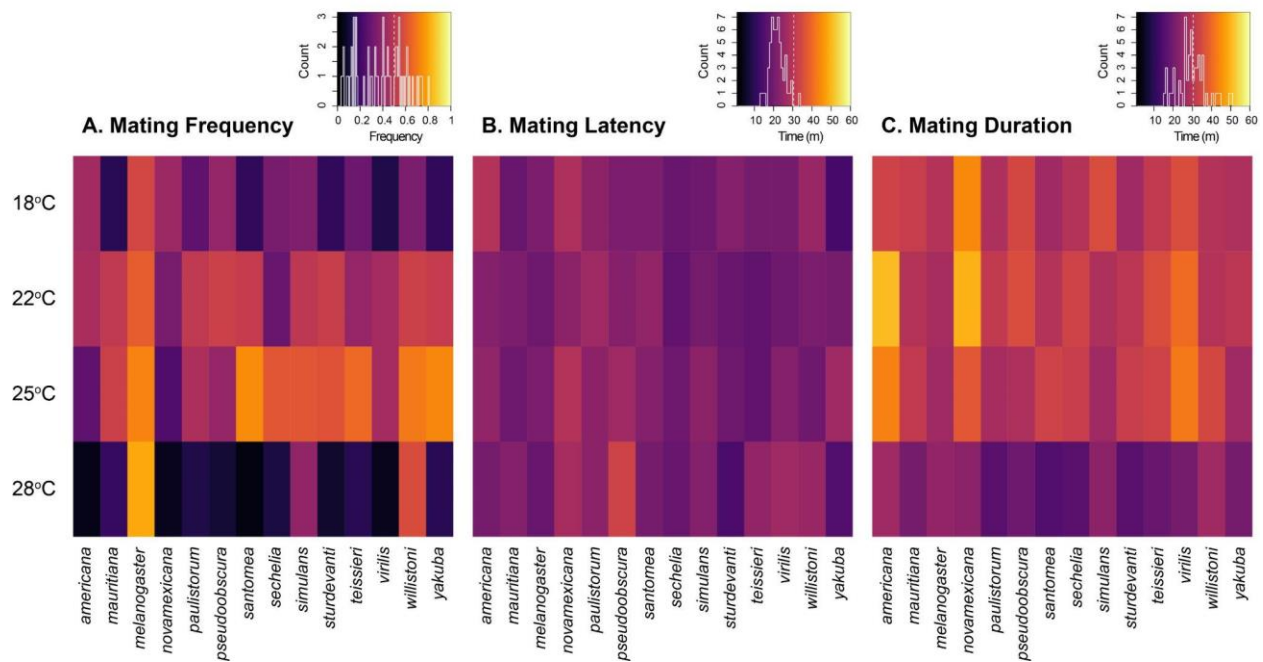
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850 **FIGURE 3. Non-choice individual experiments show strong differences in**
851 **conspecific mating propensity at different temperatures. A.** Mating frequency in
852 conspecific mating trials of 14 different species of *Drosophila* measured as a proportion
853 of successful matings over total trials. **B.** Mating latency in the same mating trials,
854 measured as the time (in minutes) from the beginning of the trial to the onset of
855 copulation. **C.** Mating duration in the individual trials, measured as the time (minutes)
856 from mounting to separation.

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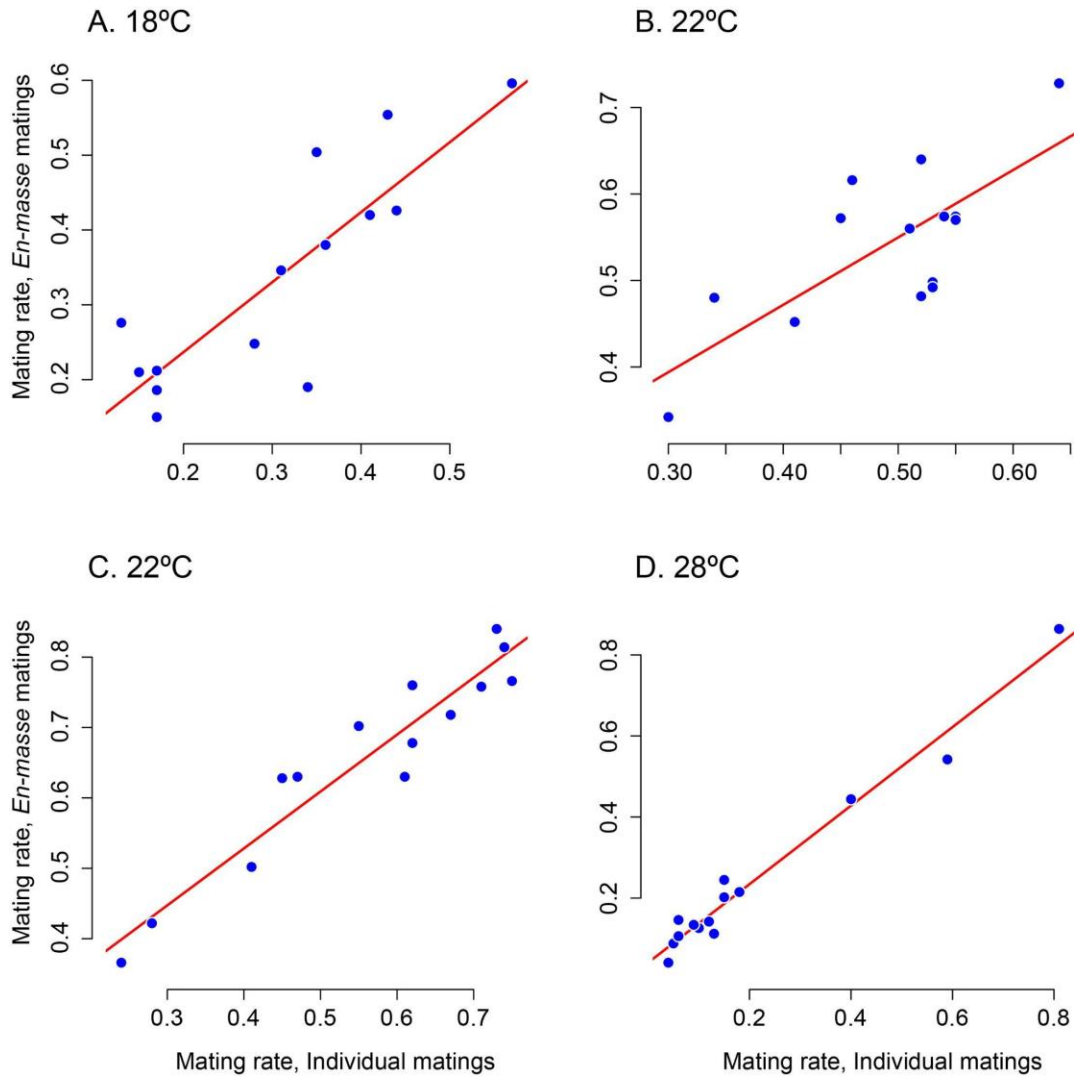


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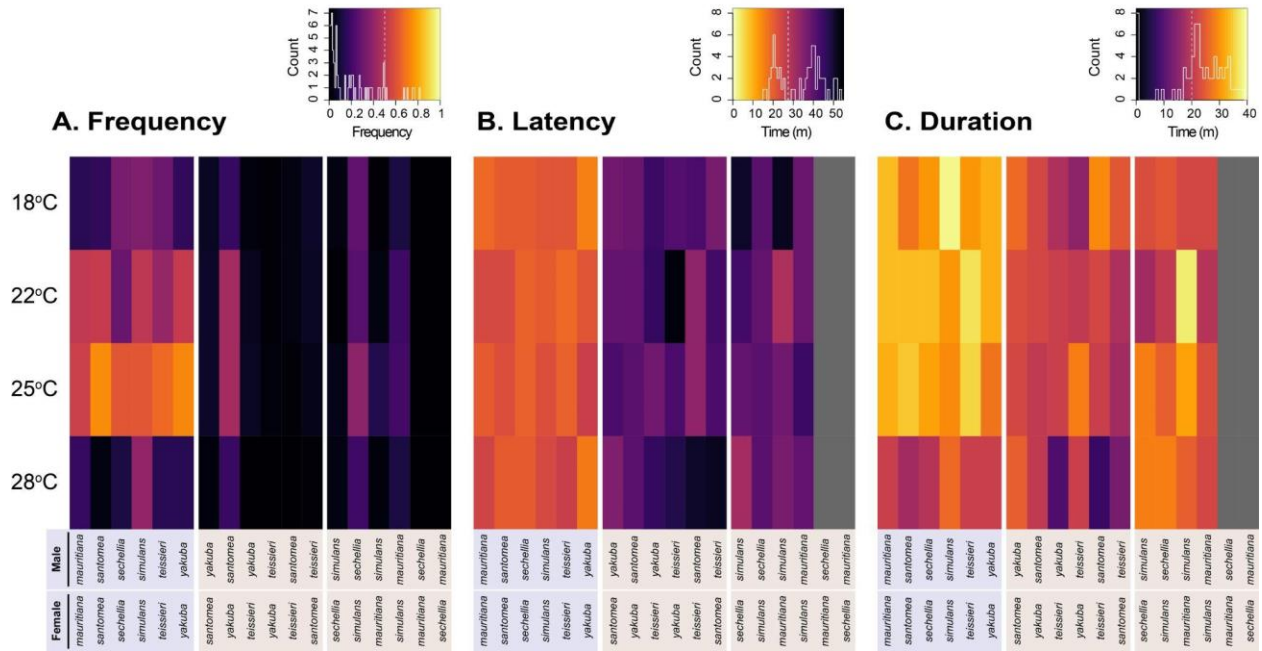
861 **FIGURE 4. Mating rates are highly correlated between *en-masse* and individual**
862 **non-choice experiments in conspecific matings.** The four panels show correlations
863 at four different temperatures. **A. 18°C. B. 28°C. C. 25°C. D. 28°C.**
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866

867 **FIGURE 5. Non-choice individual mating trials reveal a small effect of temperature**
 868 **in heterospecific mating propensity.** A subset of the conspecific crosses shown in
 869 Figure 2 is shown for comparison. **A.** Mating frequency measured as a proportion of
 870 successful matings over total trials for two *Drosophila* species triads. **B.** Mating latency
 871 measured in minutes. **C.** Mating duration measured in minutes.
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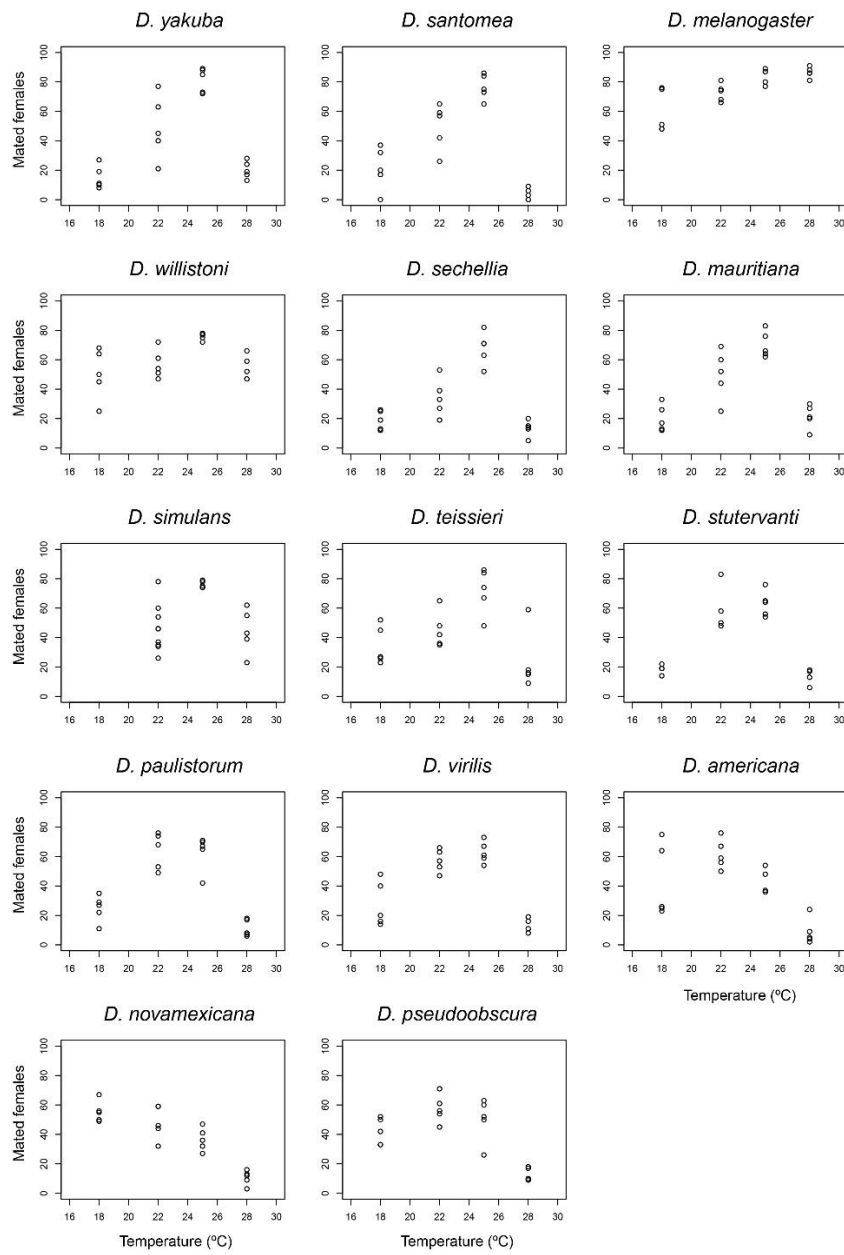
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885 Supplemental Material

886 Supplementary Figures

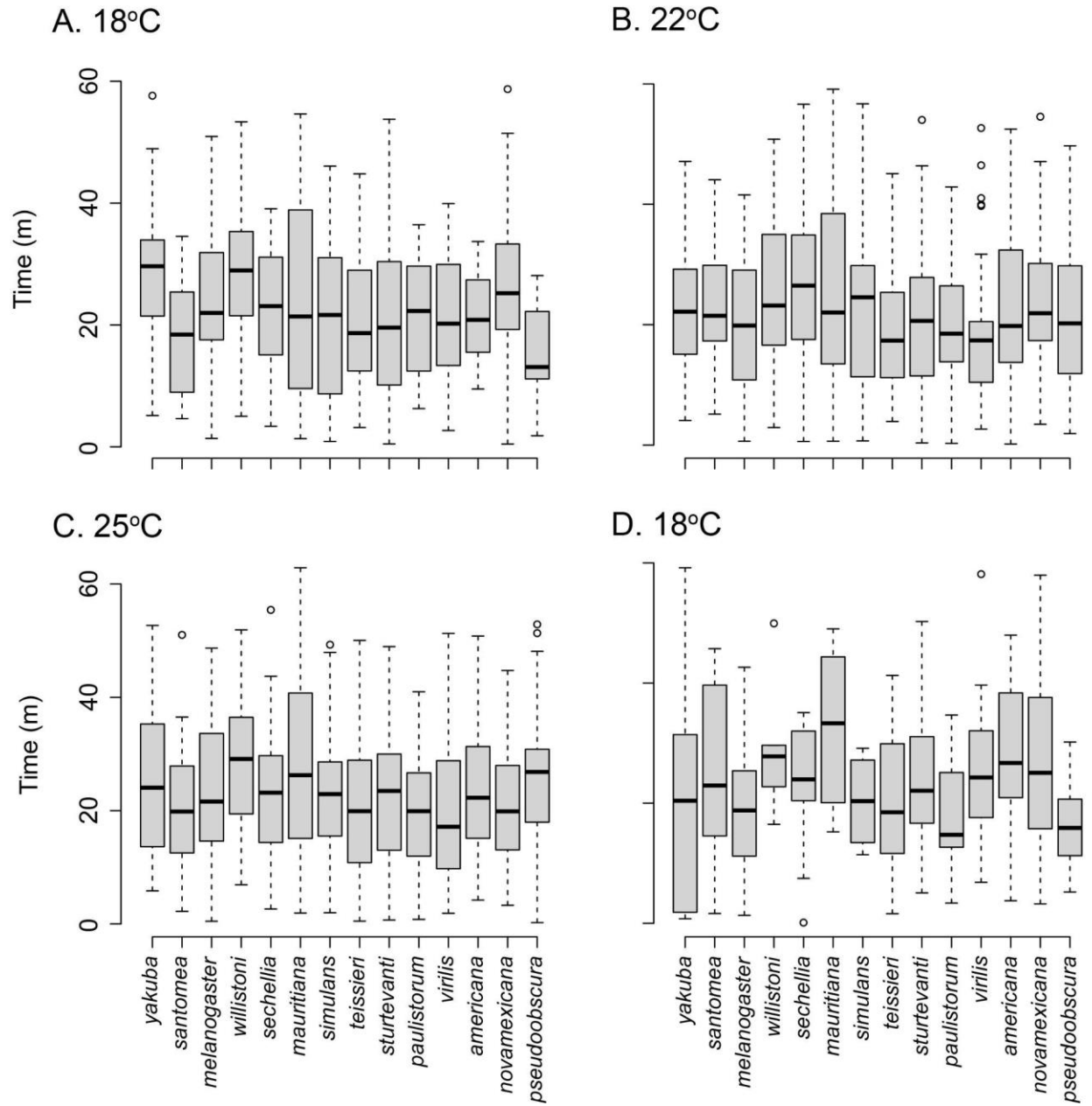
887 **FIGURE S1. Proportion of females mated for each of the fourteen species included in this**
888 **study.** Note that unlike the linear models we present in the text which had a binomial response
889 (mated vs. unmated), each point in these panels show the proportion of mated females in a
890 replicate.



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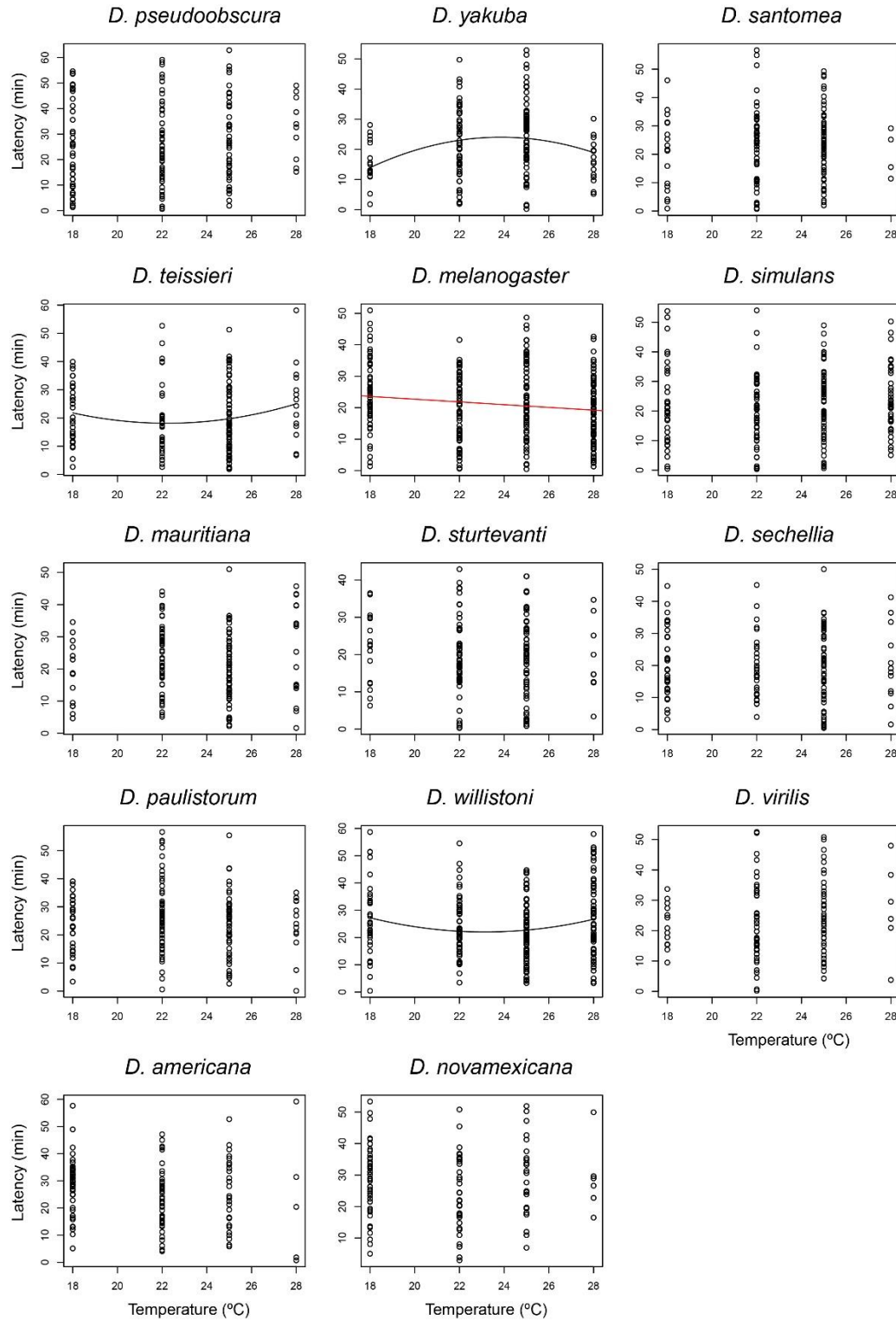
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893 **FIGURE S2.** Conspecific mating latency during the individual experiments at four temperatures.
894



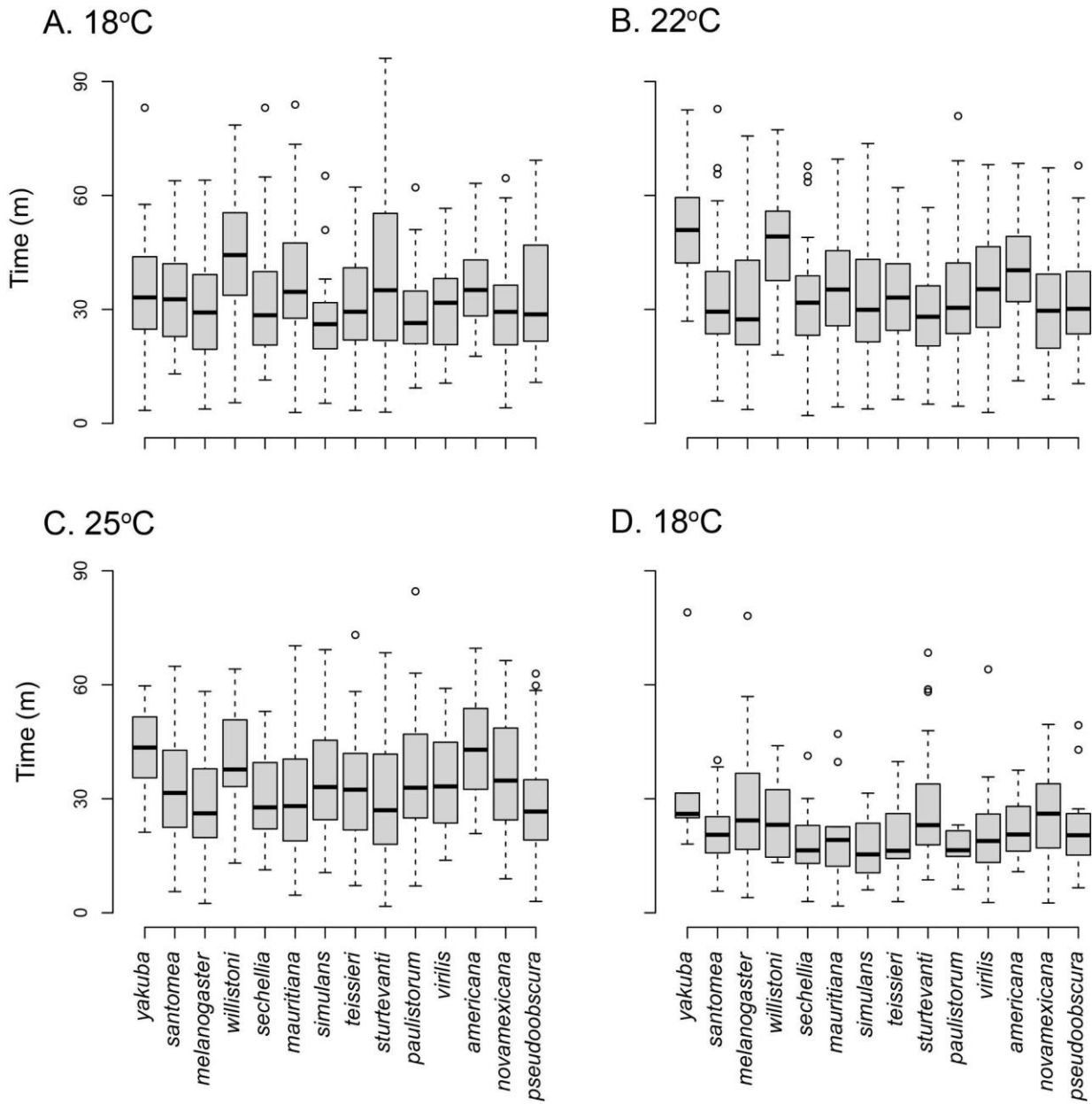
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898 **FIGURE S3.** Conspecific mating latency shown for each species along the assayed temperature
899 range.
900



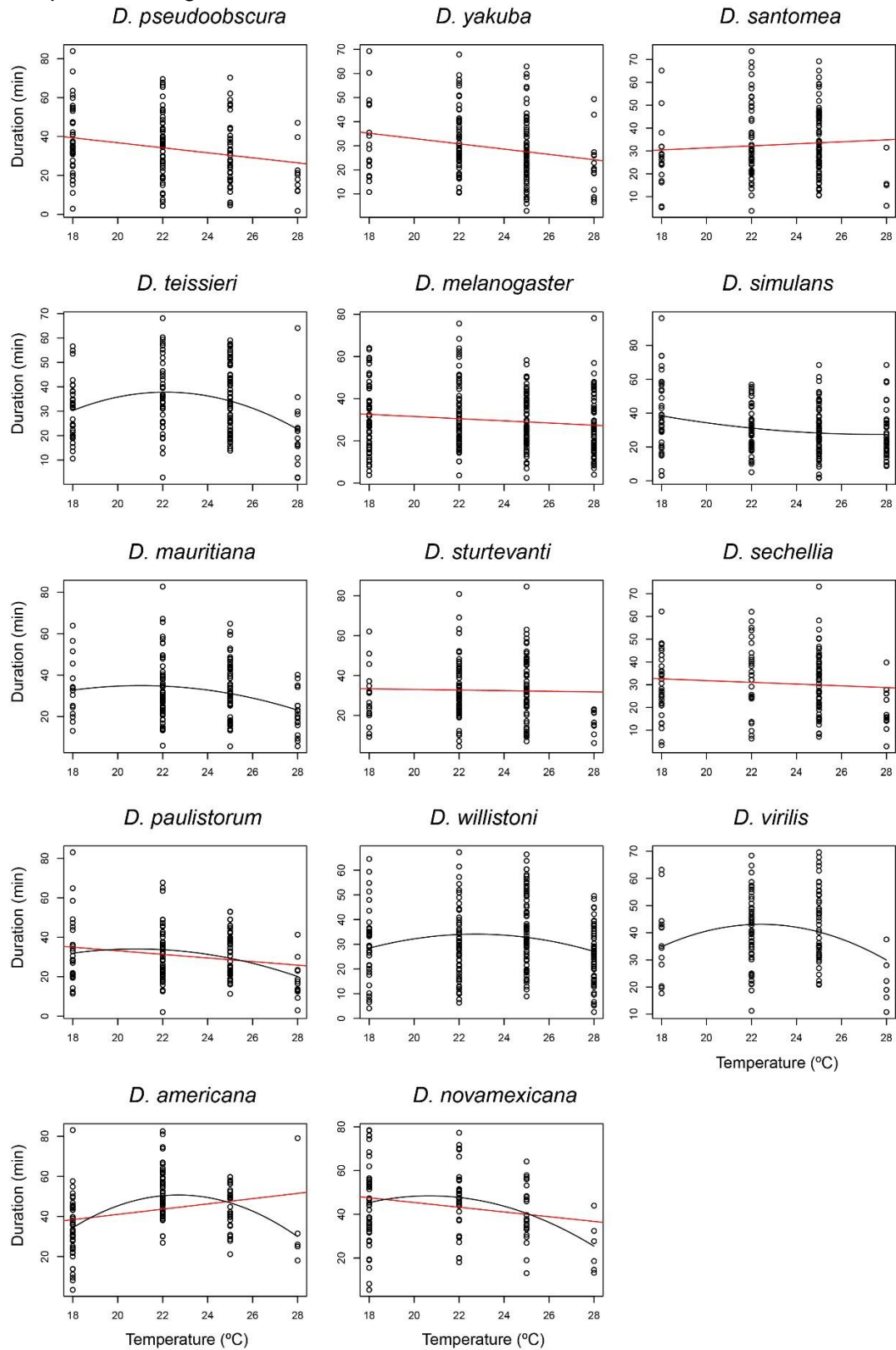
901

902 **FIGURE S4.** Conspecific mating duration during the individual experiments at four
903 temperatures.
904



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908 **FIGURE S5.** Conspecific mating duration shown for each species along the assayed
909 temperature range.



910

911 **Supplementary Tables**

912 **TABLE S1. Isofemale lines used in this study.**

Species	Isofemale line	Collector	Location	Year
<i>D. sechellia</i>	Anro105	Matute, D.R.	Anse Royale, La Digue, Seychelles	2012
<i>D. simulans</i>	MP2.45	Matute, D.R.	Mpala, Kenya	2018
<i>D. santomea</i>	TNE1500.2	Matute, D.R.	Bom sucesso, São Tomé é Príncipe	2015
<i>D. yakuba</i>	BOSU1151	Matute, D.R.	San Nicolau, São Tomé é Príncipe	2015
<i>D. teissieri</i>	NM5.1	Matute, D.R.	Bioko, Equatorial Guinea	2009
<i>D. melanogaster</i>	Loreto	Matute, D.R.	Bioko, Equatorial Guinea	2009
<i>D. mauritiana</i>	Standard	Kitagawa, O.	Mauritius	1981
<i>D. pseudoobscura</i>	14011-0121.117	Mateos, M.	Tucson, Arizona	2004
<i>D. novamexicana</i>	15010-1031.14	Castrezana, S.	Moab, Utah	1949
<i>D. americana</i>	15010-0951.22	McAllister, B.	Illinois River at Duck Island, Illinois	2005
<i>D. virilis</i>	15010-1051.00	NA	Pasadena, California.	Before 1984
<i>D. willistoni</i>	14030-0811.32	Bruck, J.	Monkey Hill, St. Kitts,	2005
<i>D. paulistorum</i>	14030-0771.00	NA	Copan, Honduras.	Before 1984
<i>D. sturtevantii</i>	14043-0871.01	Heed, W.	Bucaramanga, Colombia	1956

913 **TABLE S2. Interspecific hybridizations included in this study.**

Mother	Father
<i>D. yakuba</i>	<i>D. santomea</i>
<i>D. santomea</i>	<i>D. yakuba</i>
<i>D. yakuba</i>	<i>D. teissieri</i>
<i>D. teissieri</i>	<i>D. yakuba</i>
<i>D. santomea</i>	<i>D. teissieri</i>
<i>D. teissieri</i>	<i>D. yakuba</i>
<i>D. simulans</i>	<i>D. sechellia</i>
<i>D. sechellia</i>	<i>D. simulans</i>
<i>D. sechellia</i>	<i>D. mauritiana</i>
<i>D. sechellia</i>	<i>D. sechellia</i>
<i>D. mauritiana</i>	<i>D. simulans</i>
<i>D. simulans</i>	<i>D. mauritiana</i>

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916

917 **TABLE S3. Akaike Information Criterion (AIC) values for linear logistic and quadratic**
918 **logistic models to study the effect of temperature and cross direction in heterospecific**
919 **mating frequency in *en-masse* mating experiments.**

Species pair	AIC_{Linear}	AIC_{Quadratic}
<i>D. yakuba/D. santomea</i>	3,453.98	3,297.38
<i>D. yakuba/D. teissieri</i>	968.34	941.56
<i>D. santomea/D. teissieri</i>	1,524.44	1,505.53
<i>D. simulans/D. sechellia</i>	3,089.12	3,083.27
<i>D. simulans/D. mauritiana</i>	2,837.22	2,809.49
<i>D. sechellia/D. mauritiana</i>	698.25	702.11

920

921

922 **TABLE S4. Linear logistic models suggest that temperature has a moderate effect on**
 923 **mating propensity in six types of heterospecific *en-masse* matings.** All the likelihood ratio
 924 tests (LRT) comparisons involve one degree of freedom.

Species pair	Direction		Temperature		Direction x temperature	
	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
<i>D. yakuba</i> / <i>D. santomea</i>	437.567	< 1 ×10 ⁻¹⁰	1.0658	0.302	9.120	0.003
<i>D. yakuba</i> / <i>D. teissieri</i>	43.888	< 1 ×10 ⁻¹⁰	2.396	0.122	0.187	0.666
<i>D. santomea</i> / <i>D. teissieri</i>	37.072	1.139×10 ⁻⁹	11.175	8.290×10⁻⁴	3.039 ×10 ⁻⁴	0.986
<i>D. simulans</i> / <i>D. sechellia</i>	779.092	< 1 ×10 ⁻¹⁰	0.167	0.683	7.862	0.005
<i>D. simulans</i> / <i>D. mauritiana</i>	388.847	< 1 ×10 ⁻¹⁰	2.495	0.114	2.780	0.094
<i>D. sechellia</i> / <i>D. mauritiana</i>	0.118	0.732	4.067	0.044	0.687	0.407

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927 **TABLE S5. Correlation test of mating frequencies between *en-masse* and individual non-**
928 **choice experiments.**

Temperature (°C)	Pearson's product-moment correlation	<i>P</i> -value
18	0.848	1.281×10^{-4}
22	0.749	2.044×10^{-3}
25	0.953	1.497×10^{-7}
28	0.987	7.783×10^{-11}

929

930

931 **TABLE S6. AIC values and effect significance (LRT results) for linear logistic and**
 932 **quadratic logistic models to study the effect of temperature and cross direction in**
 933 **heterospecific mating frequency in individual non-choice mating experiments.**

Species	Quadratic Model			Linear Model	AIC
	$I(\text{temperature}^2)$	Temperature	AIC	Temperature	
<i>D. pseudoobscura</i>	$\chi^2_1 = 28.419$ $P = 9.773e^{-08}$	$\chi^2_1 = 24.942$ $P = 5.907e^{-07}$	509.9356	$\chi^2_1 = 21.541$ $P = 3.463e^{-06}$	508.5483
<i>D. yakuba</i>	$\chi^2_1 = 111.15$ $P < 2.2e^{-16}$	$\chi^2_1 = 112.64$ $P < 1 \times 10^{-10}$	471.1564	$\chi^2_1 = 1.5657$ $P = 0.2108$	540.0222
<i>D. santomea</i>	$\chi^2_1 = 160.45$ $P < 2.2e^{-16}$	$\chi^2_1 = 159.45$ $P < 1 \times 10^{-10}$	425.9435	$\chi^2_1 = 0.021533$ $P = 0.8833$	532.1967
<i>D. teissieri</i>	$\chi^2_1 = 39.629$ $P = 3.071e^{-10}$	$\chi^2_1 = 38.839$ $P = 4.602e^{-10}$	528.3322	$\chi^2_1 = 0.50133$ $P = 0.4789$	536.6658
<i>D. melanogaster</i>	$\chi^2_1 = 0.159706$ $P = 0.6894$	$\chi^2_1 = 0.018713$ $P = 0.8912$	425.9435	$\chi^2_1 = 15.165$ $P = 9.854e^{-05}$	532.1967
<i>D. simulans</i>	$\chi^2_1 = 12.561$ $P = 0.0003938$	$\chi^2_1 = 13.102$ $P = 0.0002949$	573.4243	$\chi^2_1 = 1.5361$ $P = 0.2152$	555.771
<i>D. mauritiana</i>	$\chi^2_1 = 68.146$ $P < 2.2e^{-16}$	$\chi^2_1 = 69.123$ $P < 1 \times 10^{-10}$	486.3818	$\chi^2_1 = 1.1626$ $P = 0.2809$	520.7947
<i>D. sturtevantii</i>	$\chi^2_1 = 106.33$ $P < 2.2e^{-16}$	$\chi^2_1 = 105.55$ $P < 1 \times 10^{-10}$	457.2496	$\chi^2_1 = 0.042049$ $P = 0.8375$	523.1424
<i>D. sechellia</i>	$\chi^2_1 = 20.611$ $P = 5.626e^{-06}$	$\chi^2_1 = 19.646$ $P = 9.321e^{-06}$	525.9057	$\chi^2_1 = 2.0594$ $P = 0.1513$	517.3777
<i>D. paulistorum</i>	$\chi^2_1 = 42.731$ $P = 6.280e^{-11}$	$\chi^2_1 = 40.804$ $P = 1.683e^{-10}$	506.288	$\chi^2_1 = 3.711$ $P = 0.05405$	518.2463
<i>D. willistoni</i>	$\chi^2_1 = 8.4439$ $P = 0.003663$	$\chi^2_1 = 10.1216$ $P = 0.001465$	425.9435	$\chi^2_1 = 17.224$ $P = 3.321e^{-05}$	532.1967
<i>D. virilis</i>	$\chi^2_1 = 74.908$ $P < 2.2e^{-16}$	$\chi^2_1 = 73.927$ $P < 1 \times 10^{-10}$	426.4345	$\chi^2_1 = 0.27382$ $P = 0.6008$	472.3098
<i>D. americana</i>	$\chi^2_1 = 11.7211$ $P = 0.000618$	$\chi^2_1 = 8.8163$ $P = 0.002986$	470.7596	$\chi^2_1 = 40.809$ $P = 1.679e^{-10}$	456.86
<i>D. novamexicana</i>	$\chi^2_1 = 2.1733$ $P = 0.1404$	$\chi^2_1 = 1.1047$ $P = 0.2932$	450.7751	$\chi^2_1 = 37.175$ $P = 1.08e^{-09}$	431.4303

934 **TABLE S7.** Effect of temperature on mating latency in conspecific crosses for 14 different
 935 species of *Drosophila*.

Species	Quadratic Model			Linear Model	
	I(temperature ²)	Temperature	AIC _{Quadratic}	Temperature	AIC _{Linear}
<i>D. pseudoobscura</i>	$F_{1,144} = 0.265,$ $P = 0.608$	$F_{1,144} = 0.164,$ $P = 0.686$	1241.225	$F_{1,145} = 2.375,$ $P = 0.125$	-616.75
<i>D. yakuba</i>	$F_{1,156} = 9.366,$ $P = 0.003$	$F_{1,156} = 10.142,$ $P = 0.002$	1216.555	$F_{1,157} = 3.022,$ $P = 0.084$	-608.91
<i>D. santomea</i>	$F_{1,146} = 0.728,$ $P = 0.395$	$F_{1,146} = 0.766,$ $P = 0.383$	1165.271	$F_{1,147} = 0.131,$ $P = 0.718$	-579.01
<i>D. teissieri</i>	$F_{1,151} = 4.398,$ $P = 0.0377$	$F_{1,151} = 4.288,$ $P = 0.040$	1199.973	$F_{1,151} = 0.091,$ $P = 0.763$	-598.20
<i>D. melanogaster</i>	$F_{1,272} = 0.005,$ $P = 0.942$	$F_{1,272} = 0.054,$ $P = 0.816$	2100.766	$F_{1,273} = 5.845,$ $P = 0.016$	-1046.4
<i>D. simulans</i>	$F_{1,186} = 0.610,$ $P = 0.436$	$F_{1,186} = 0.498,$ $P = 0.481$	1476.858	$F_{1,187} = 1.067,$ $P = 0.303$	-734.74
<i>D. mauritiana</i>	$F_{1,137} = 0.015,$ $P = 0.904$	$F_{1,137} = 0.034,$ $P = 0.854$	1074.259	$F_{1,138} = 0.721,$ $P = 0.397$	-533.14
<i>D. sturtevantii</i>	$F_{1,138} = 0.119,$ $P = 0.731$	$F_{1,138} = 0.163,$ $P = 0.687$	1062.052	$F_{1,139} = 0.678,$ $P = 0.412$	-527.09
<i>D. sechellia</i>	$F_{1,135} = 0.353,$ $P = 0.554$	$F_{1,135} = 0.397,$ $P = 0.530$	1055.877	$F_{1,136} = 0.341,$ $P = 0.560$	-524.12
<i>D. paulistorum</i>	$F_{1,137} = 1.548,$ $P = 0.216$	$F_{1,137} = 1.454,$ $P = 0.230$	1089.785	$F_{1,138} = 0.231,$ $P = 0.632$	-541.68
<i>D. willistoni</i>	$F_{1,217} = 6.925,$ $P = 0.009$	$F_{1,217} = 6.856,$ $P = 0.009$	1731.497	$F_{1,218} = 0.009,$ $P = 0.926$	-865.20
<i>D. virilis</i>	$F_{1,106} = 0.212,$ $P = 0.646$	$F_{1,106} = 0.151,$ $P = 0.698$	858.1922	$F_{1,107} = 0.961,$ $P = 0.329$	-425.21
<i>D. americana</i>	$F_{1,120} = 1.209,$ $P = 0.274$	$F_{1,120} = 1.439,$ $P = 0.233$	999.4032	$F_{1,121} = 2.364,$ $P = 0.127$	-475.70
<i>D. novamexicana</i>	$F_{1,104} = 1.845,$ $P = 0.177$	$F_{1,104} = 1.840,$ $P = 0.178$	834.4479	$F_{1,105} = 3e^{-04},$ $P = 0.987$	-414.16

937 **TABLE S8. Linear and quadratic logistic models suggest that temperature has a**
 938 **moderate effect in the duration of some conspecific matings.**

Species	Quadratic Model		AIC _{Quadratic}	Linear Model	AIC _{Linear}
	I(temperature ²)	Temperature		Temperature	
<i>D. pseudoobscura</i>	$F_{1,144} = 1.826,$ $P = 0.179$	$F_{1,156} = 1.312,$ $P = 0.254$	1249.029	$F_{1,144} = 8.215,$ $P = 0.005$	1248.882
<i>D. yakuba</i>	$F_{1,156} = 1.546,$ $P = 0.2156$	$F_{1,156} = 1.082,$ $P = 0.300$	1285.06	$F_{1,157} = 7.190,$ $P = 0.008$	1285.492
<i>D. santomea</i>	$F_{1,146} = 4.739,$ $P = 0.031$	$F_{1,146} = 4.972,$ $P = 0.027$	1230.471	$F_{1,147} = 0.763,$ $P = 0.384$	1233.23
<i>D. teissieri</i>	$F_{1,151} = 14.875,$ $P = 0.0002$	$F_{1,151} = 14.399,$ $P = 0.0002$	1246.852	$F_{1,152} = 0.531,$ $P = 0.467$	1259.321
<i>D. melanogaster</i>	$F_{1,272} = 0.555,$ $P = 0.457$	$F_{1,272} = 0.360,$ $P = 0.549$	2251.452	$F_{1,273} = 4.664,$ $P = 0.032$	2250.012
<i>D. simulans</i>	$F_{1,186} = 1.282,$ $P = 0.259$	$F_{1,186} = 1.845,$ $P = 0.176$	1586.757	$F_{1,187} = 10.406,$ $P = 0.001$	1586.055
<i>D. mauritiana</i>	$F_{1,137} = 3.835,$ $P = 0.052$	$F_{1,137} = 3.159,$ $P = 0.078$	1143.715	$F_{1,138} = 5.290,$ $P = 0.023$	1145.58
<i>D. sturtevantii</i>	$F_{1,138} = 7.997,$ $P = 0.005$	$F_{1,138} = 7.827,$ $P = 0.006$	1173.91	$F_{1,139} = 0.092,$ $P = 0.763$	1179.853
<i>D. sechellia</i>	$F_{1,135} = 7.918,$ $P = 0.006$	$F_{1,135} = 7.498,$ $P = 0.007$	1112.746	$F_{1,136} = 1.107,$ $P = 0.295$	1118.611
<i>D. paulistorum</i>	$F_{1,137} = 5.894,$ $P = 0.016$	$F_{1,137} = 5.019,$ $P = 0.027$	1122.547	$F_{1,138} = 5.885,$ $P = 0.017$	1126.445
<i>D. willistoni</i>	$F_{1,217} = 8.676,$ $P = 0.004$	$F_{1,217} = 8.294,$ $P = 0.004$	1790.954	$F_{1,218} = 0.666,$ $P = 0.415$	1797.579
<i>D. virilis</i>	$F_{1,106} = 7.293,$ $P = 0.008$	$F_{1,106} = 7.169,$ $P = 0.009$	883.6012	$F_{1,107} = 0.045,$ $P = 0.832$	888.8541
<i>D. americana</i>	$F_{1,120} = 27.008,$ $P = 8.419e-07$	$F_{1,120} = 29.115,$ $P = 3.485e-07$	999.4032	$F_{1,121} = 8.218,$ $P = 0.005$	1022.371
<i>D. novamexicana</i>	$F_{1,104} = 6.281,$ $P = 0.014$	$F_{1,104} = 5.558,$ $P = 0.020$	905.669	$F_{1,105} = 4.546,$ $P = 0.035$	909.9437

940 **TABLE S9. AIC values for heterospecific linear logistic and quadratic logistic models**
941 **using the mating frequency in heterospecific individual non-choice matings.**

Species pair	AIC_{Linear}	AIC_{Quadratic}
<i>D. yakuba</i> / <i>D. santomea</i>	699.080	668.577
<i>D. yakuba</i> / <i>D. teissieri</i>	226.821	225.142
<i>D. santomea</i> / <i>D. teissieri</i>	273.291	275.879
<i>D. simulans</i> / <i>D. sechellia</i>	609.200	609.775
<i>D. simulans</i> / <i>D. mauritiana</i>	537.200	528.301
<i>D. sechellia</i> / <i>D. mauritiana</i>	NA	NA

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944 **TABLE S10. Linear logistic models suggest that temperature has a moderate effect on**
 945 **mating propensity in some of the six types of heterospecific *en-masse* matings.** The
 946 metric of isolation is receptivity of females in *en-masse* matings. All the likelihood ratio tests
 947 (LRT) comparisons involve one degree of freedom.

Species pair	Direction		Temperature		Direction × temperature	
	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
<i>D. yakuba</i> / <i>D. santomea</i>	88.465	< 1 × 10⁻¹⁰	4.114×10 ⁻⁵	0.995	1.304	0.254
<i>D. yakuba</i> / <i>D. teissieri</i>	3.420	0.064	0.258	0.611	7.882×10 ⁻⁷	0.999
<i>D. santomea</i> / <i>D. teissieri</i>	82.65	< 1 × 10⁻¹⁰	5.843	0.016	4.961×10 ⁻³	0.944
<i>D. simulans</i> / <i>D. sechellia</i>	98.171	< 1 × 10⁻¹⁰	0.183	0.669	0.260	0.610
<i>D. simulans</i> / <i>D. mauritiana</i>	23.387	1.324 × 10⁻⁶	0.812	0.368	1.098	0.295
<i>D. sechellia</i> / <i>D. mauritiana</i>	0.58!	0.446	3.9648	0.04646	0.687	0.407

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950 **TABLE S11. AIC values for linear and quadratic models studying the effect of**
951 **temperature and reciprocal mating direction on mating latency in five heterospecific**
952 **matings.**

Species pair	AIC_{Linear}	AIC_{Quadratic}
<i>D. yakuba</i> / <i>D. santomea</i>	1,168.930	1,172.186
<i>D. yakuba</i> / <i>D. teissieri</i>	194.144	197.807
<i>D. santomea</i> / <i>D. teissieri</i>	257.443	258.268
<i>D. simulans</i> / <i>D. sechellia</i>	992.774	996.625
<i>D. simulans</i> / <i>D. mauritiana</i>	677.286	678.923
<i>D. sechellia</i> / <i>D. mauritiana</i>	NA	NA

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955 **TABLE S12. AIC values for linear and quadratic models studying the effect of**
956 **temperature and reciprocal mating direction on mating duration in five heterospecific**
957 **matings.**

Species pair	AIC_{Linear}	AIC_{Quadratic}
<i>D. yakuba</i> / <i>D. santomea</i>	1,231.755	1,235.519
<i>D. yakuba</i> / <i>D. teissieri</i>	207.900	211.006
<i>D. santomea</i> / <i>D. teissieri</i>	253.632	257.381
<i>D. simulans</i> / <i>D. sechellia</i>	1,018.952	1,020.806
<i>D. simulans</i> / <i>D. mauritiana</i>	707.080	709.014
<i>D. sechellia</i> / <i>D. mauritiana</i>	NA	NA

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