Title: Long-sperm precedence and other cryptic female choices in *Drosophila melanogaster*

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Data availability statement

The data and code underlying this article are available in the Dryad Digital Repository, at https://dx.doi.org/[doi].

Author contributions

BP and LCM conceived and designed the experiment. BP performed the experiment and statistical analyses. JYY performed the chemical analysis. BP and LCM wrote the paper. All authors read and provided feedback on the manuscript.

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Conflict of interest statement

The authors have no conflict of interest to declare.

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LONG-SPERM PRECEDENCE AND OTHER CRYPTIC FEMALE CHOICES IN *DROSOPHILA MELANOGASTER*

Abstract

Females that mate multiply make postmating choices about which sperm fertilize their eggs (cryptic female choice); however, the male characteristics they use to make such choices remain unclear. In this study, we sought to understand female sperm use patterns by evaluating whether *Drosophila melanogaster* females adjust sperm use (second male paternity) in response to four main factors: male genotype, male courtship effort, male pheromone alteration, and male postmating reproductive morphology. Our experiment was replicated across four different *D. melanogaster* lines, in a full factorial design, including a pheromone manipulation in which second males were perfumed to resemble heterospecific (*D. yakuba*) males. We found that females prefer longer sperm—regardless of mating order—in almost all contexts; this observed pattern of 'long-sperm precedence' is consistent with female postcopulatory choice of high-fitness male traits. Nonetheless, we also found that this general preference can be plastically altered by females in response to effects including perfuming treatment; this differential female sperm use is between otherwise identical males, and therefore solely female-mediated. Furthermore, our finding that females exercise choice using diverse criteria suggests a possible mechanism for the maintenance of variation in sexually selected male traits.

Keywords: cryptic female choice, sperm, pheromones, *Drosophila*

Teaser text

How do females make choices about which sperm will fertilize their eggs? In this study, we assess four geographically diverse *Drosophila melanogaster* lines to show that females strongly prefer long sperm—a pattern consistent with female preference for high-fitness males. In addition, we perfumed males to show that females can adjust this general preference based on other factors, including pheromones. Because this adjustment occurs between genotypically identical males, it is entirely female-mediated.

1 Introduction

Reproduction in nature is costly; organisms are therefore expected to use their reproductive resources judiciously and to adjust them according to reproductive context. Examples of such behavioral reproductive plasticity span diverse taxa, from palm trees (Barot et al. 2005) to guppies (Gasparini and Evans 2018) to *Drosophila* (Nandy et al. 2016). One specific strategy for plastically allocating reproductive resources is to adjust choices about reproductive partners. Females, in particular, are known to do this in numerous ways prior to mating. For instance, in red jungle fowl, females choose to mate with males with brighter feathers and longer tails; these traits are more prominent in unparasitized males and are therefore honest indicators of male health (Zuk et al. 1990). While many such cases involve choices exercised before mating, some organisms also have the ability to selectively allocate postmating reproductive resources; for instance, female guppies fertilize more eggs when mating with more colorful focal males in comparison to stimulus males (Pilastro et al. 2004). However, because most of these choices are made during and after mating events, it remains hard to differentiate the relative contributions of

each mating partner, the criteria on which their choices are based, and therefore the degree to which these choices could be adaptive.

Interestingly, in *Drosophila*, postmating reproductive plasticity has primarily been studied in males. Even under optimal conditions, *Drosophila melanogaster* males are known to be ejaculate-limited and cannot produce progeny after two to five matings in a day (Douglas et al. 2020). Plastic allocation of reproductive resources could therefore allow them to prioritize sperm and seminal fluid for preferred mates. Consistent with this, *D. melanogaster* males use premating information, such as female condition and the relative abundance of potential sexual competitors, to modulate ejaculate composition (Sirot 2019, Wigby et al. 2009, Lüpold et al. 2011, Sirot et al. 2011, Garbaczewska et al. 2013). *D. melanogaster* males also allocate more resources to matings with large females—an investment that reduces their success at defensive sperm competition in subsequent matings (Anastasio et al. 2023). Together, these studies broadly indicate that costs to *Drosophila* males drive plasticity in their reproductive allocation during mating, as well as identify specific criteria used in making these behavioral decisions.

Like males, females are also expected to selectively allocate reproductive resources (i.e., eggs) at postmating stages, through the action of "cryptic female choice"—the female-mediated physical and chemical factors that determine which sperm are used to fertilize eggs after mating (Firman et al. 2017). In *Drosophila,* several proximate mechanisms of cryptic female choice involve modulating the storage of sperm prior to fertilization. *Drosophila* females have two types of sperm storage organs and can bias sperm use through differential sperm storage and/or by preferentially drawing sperm from these alternative organs (Manier et al. 2013b). Females can also manipulate the identity of sperm available in storage by adjusting the time until sperm ejection after specific matings (Snook and Hosken 2004, Manier et al. 2013a, Laturney et al.

114 2018). Finally, it has been proposed that the size of sperm storage organs themselves might
115 provide a mechanism by which females make these choices. For example, females
116 experimentally evolved to have longer seminal receptacles have been shown to select for males
117 with longer sperm (Miller and Pitnick 2002). Even when the specific mechanism is unknown, it
118 is evident that *Drosophila* females can change sperm use patterns depending upon context,
119 including according to male genotype identity (Chow et al. 2010). What is comparatively poorly
120 understood, however, is the evolutionary explanation for these apparent female choices,
121 including the criteria used by females to exercise them.

122 One reason for this is the challenge of differentiating female choice from primarily male-
123 mediated factors that can also influence the outcome of postmating processes (Firman et al.
124 2017). For instance, *Drosophila simulans* males that are more attractive to females also sire more
125 offspring as second males; however, it is unclear whether this is because females preferentially
126 use sperm from attractive males, or because attractive males are intrinsically better sperm
127 competitors (Hosken et al. 2008). While the former explanation would be consistent with an
128 adaptive function for cryptic female choice, the latter explanation implies passive female
129 responses to the outcome of male-male sexual selection. One strategy for determining the
130 specific contribution of female choices to patterns of biased sperm use is to evaluate how these
131 patterns change when female perception of male identity is manipulated, without influencing
132 male identity itself. Although female assessment of male identity is likely to be complex, in
133 *Drosophila,* female premating preference primarily depends on auditory and chemosensory cues
134 (Yamamoto and Nakano 1999, Greenspan and Ferveur 2000); manipulating these cues could
135 therefore modify this assessment. Contact pheromones are one such cue. In insects broadly, and
136 flies specifically, these pheromones include cuticular hydrocarbons (CHCs)—waxy compounds

137 on the outer surface (cuticle) of many insects that can be used as sexual pheromones in mate
138 choice by males (Billeter et al. 2009) and females (Scott 1994), both within and between species
139 (Blows and Allan 1998). CHC composition can be artificially altered to assess its effect on mate
140 choice by "perfuming" focal males with the CHCs of other male genotypes (Dyer et. al. 2014,
141 Wang et al. 2011). Perfuming males with CHCs from unfamiliar or unpreferred males,
142 immediately prior to mating, can therefore manipulate female perception of male identity while
143 keeping male postmating quality the same.

144 In this study, we sought to understand the nature and relative importance of factors that
145 females use when exercising cryptic female choice. To do so, we evaluated whether *Drosophila*
146 *melanogaster* females adjust their sperm use patterns in response to four main factors: male
147 genotype, male courtship effort, male pheromone alteration, and male postmating reproductive
148 morphology. We manipulated male pheromones via "perfuming" with heterospecific (*D. yakuba*)
149 males—thereby changing the perceived, but not actual, identity of these treatment males. We also
150 evaluated postmating reproductive trait variation in males and females (testis length, sperm
151 length, male reproductive tract [MRT] mass, sperm storage organ size, female reproductive tract
152 [FRT] mass) to assess their contribution to sperm preference patterns. We assessed how second-
153 male paternity (proportion of offspring sired) changed according to each factor, by mating males
154 of each type to females that had previously mated with a consistent first-male tester genotype.
155 Our experiment was replicated across four different *D. melanogaster* lines in a full factorial
156 design, to evaluate whether the effects of these factors on sperm use depend upon female
157 genotype, male genotype, or their interaction. We find clear evidence that females adjust their
158 use of sperm from the second male. In particular, females show a general preference for longer
159 sperm, whether that sperm is from the first or the second male—i.e. 'long-sperm precedence'

rather than ‘last-male sperm precedence’ (Laturney et al. 2018). Nonetheless, we also find that this general preference can be overridden in specific contexts by female-mediated changes in response to male genotype, courtship effort, and perfuming treatment. The presence and direction of these cryptic choices vary in complex ways, including evidence that females of some genotypes adjust sperm use based on their perception of male identity, regardless of actual identity. These data allow us to draw broader inferences about the possible criteria for, as well as the evolutionary causes and consequences of, such choices.

2 Methods

2.1 Fly stocks

All stocks were reared on standard media prepared by the Bloomington Drosophila Stock Center and kept at room temperature (~22°C). For this experiment we used four lines from among the 15 *D. melanogaster* founder lines in the Drosophila Synthetic Population Resource (DSPR) (King et al. 2012). These fully-sequenced founder lines represent a diverse collection of genotypes from different locations around the world, and are known to vary in reproductive traits (i.e., male courtship song, see Pischedda et al. 2014a). Our four DSPR founder lines (A5 [Greece], A7 [Taiwan], B4 [California], and B7 [Malaysia]) were used as focal genotypes for females and second males in each manipulation. The GFP-labeled *D. melanogaster* line (32170) was used as the first tester male. The *D. yakuba* line yak_S09_L38 was the heterospecific donor male for the heterospecific-perfumed treatment (collected by B. Cooper in Sao Tome in 2009). For each conspecific-perfumed treatment, target males were perfumed (see methods below) with males from their own genotype.

183 *2.2 Experimental design*

184 To assess patterns of preferential sperm use, including after remating with conspecific-
185 vs. heterospecific-perfumed males, we mated *D. melanogaster* females sequentially to two
186 males: first, to *D. melanogaster* GFP-labeled tester males, and second, to *D. melanogaster* target
187 males. Using a GFP-labeled first male allowed us to assess paternity share (first [tester] versus
188 second [target] males) of offspring from each female) by examining the phenotypes of offspring
189 generated after the second mating. Any offspring not expressing GFP are sired by the second
190 focal male. For our CHC manipulation, the second, target males were either perfumed by
191 genotypically identical males (conspecific-perfumed control) or perfumed by heterospecific *D.*
192 *yakuba* males (Figure 1).

193 We replicated this experimental procedure across all four *D. melanogaster* genotypes, in
194 a full factorial design. For each line, we set up matings between females and second males from
195 each of the four DSPR lines, including males from the females' own genotype. This created 16
196 cross types in total. For each cross-type, we assessed paternity share from crosses with
197 conspecific-perfumed males and with heterospecific-perfumed males. Each of these cross types
198 was repeated until successfully replicated at least five times, resulting in over 160 total crosses.

199

200 *2.3 Perfuming manipulation*

201 Males used in the second mating were perfumed in the morning before setting up crosses.
202 To do so, we vortexed four target males with 30 donor males, either of the same *D. melanogaster*
203 genotype (for conspecific-perfumed control crosses) or with 30 heterospecific (*D. yakuba*) donor
204 males (for heterospecific-perfumed treatment crosses). The flies were vortexed on medium
205 strength for one minute total, in three 20-second increments with 20-second breaks in between

206 (Pischedda et al. 2014b). The target males were separated immediately after vortexing and
207 isolated individually in vials. They were allowed to recover for one hour before setting up
208 crosses.
209
210 *2.4 Paternity assay*
211 *D. melanogaster* males were isolated and aged for seven days, while females were
212 isolated and aged for two days before setting up the first cross with a GFP-labeled tester male.
213 We allowed mating for 24 hours before removing the males. We allowed the isolated female to
214 lay eggs for an additional 24 hours and verified successful matings by observing larval activity.
215 Unmated females (those that failed to produce larvae after the first mating) were removed from
216 the experiment. Mated females were then moved to a new vial to be paired with the second male.
217 To determine whether and the degree to which females exhibit premating discrimination against
218 heterospecific-perfumed males, we observed a subset of crosses for two hours to observe the
219 number of second matings within this period and recorded the time until remating (latency) and
220 copulation duration for each. Regardless of whether matings were observed in the first two
221 hours, seven-day-old second males were housed with females for 24 hours. Males were then
222 removed and the females were transferred to a new vial. Females continued to be transferred to
223 new vials every 24 hours for three days. Progeny in these vials were allowed to mature into
224 adulthood, then counted and assessed for GFP to determine paternity (see details below). All
225 cross types were repeated until we had five successful replicates (crosses in which there is
226 evidence for both a first and second mating). Conspecific- and heterospecific-perfumed treatment
227 crosses for each genotype were completed in parallel and in a randomized order.

228 Upon eclosion, all progeny from the three post-remating vials were anesthetized with
229 CO_2 and viewed under a Leica M205FA stereo scope equipped with a UV light for visualizing
230 GFP. The presence or absence of GFP in the ocelli of the eye were used as a marker of paternity;
231 offspring with GFP ocelli were sired by the first male and were thus counted as P1. Progeny with
232 wild-type ocelli were sired by the second male and were counted as P2. If no wild-type progeny
233 were present in the three post-remating vials, we assumed that no remating event occurred, and
234 these were scored as "unremated".
235
236 *2.5 Cuticular hydrocarbon extraction and quantification*
237 We confirmed the efficacy of perfuming using gas chromatography–mass spectrometry
238 (GC/MS) analysis. We extracted cuticular hydrocarbons from males of all four genotypes, for
239 both conspecific-perfumed and heterospecific-perfumed treatments. Following treatment of
240 seven-day old virgin males, cuticular hydrocarbons were extracted from pooled samples by
241 placing five males in a 1.8 mL glass vial (Wheaton 224740 E-C Clear Glass Sample Vials) with
242 120 µL of hexane (Sigma Aldrich, St Louis, MO, USA) spiked with 10 µg/mL of hexacosane
243 (Sigma Aldrich). After 20 min, 100 µL of the solution was removed to a sterilized 1.8 mL glass
244 vial (Wheaton 224740 E-C Clear Glass Sample Vials) and allowed to evaporate under a fume
245 hood. Extracts were stored at −20°C until analysis. Five replicate samples, consisting of five flies
246 per sample (25 flies total), were prepared for each sample type.
247 We performed gas chromatography–mass spectrometry (GC/MS) analysis on a 7820A
248 GC system equipped with a 5975 Mass Selective Detector (Agilent Technologies, Inc., Santa
249 Clara, CA, USA) and a HP-5ms column ((5%-Phenyl)-methylpolysiloxane, 30 m length, 250 µm
250 ID, 0.25 µm film thickness; Agilent Technologies, Inc.). Electron ionization (EI) energy was set

251 at 70 eV. One microliter of the sample was injected in splitless mode and analyzed with helium
252 flow at 1 mL/min. We used two different temperature gradients depending on the sample type.
253 The column was set at 40°C and held for 3 min, increased to 200°C at a rate of 35°C/min, then
254 increased to 280°C at a rate of 3°C/min and held for 15 min. Chromatograms and spectra were
255 analyzed using MSD ChemStation (Agilent Technologies, Inc.). We identified CHCs based on
256 retention time and electron ionization fragmentation pattern. The abundance of each compound
257 was then quantified by normalizing the area under each CHC peak to the area of the hexacosane
258 signal using homebuilt peak selection software (personal correspondence, Dr. Scott Pletcher,
259 Univ. of Michigan).
260
261 *2.6 Postmating reproductive trait morphology*
262 We measured several elements of reproductive trait morphology in each of our lines, and
263 in the tester line, in order to assess their potential influence on patterns of female preferential
264 sperm use.
265
266 *2.6.1 Thorax length, testis length, SR length, and ST area*
267 We collected both female and male virgins and aged them to reproductive maturity (N=5
268 replicates per sex per genotype). We measured thorax length for all flies; this is a standard
269 measure of body size in *Drosophila* studies because it strongly correlates with the size of other
270 characters such as wing length (Robertson and Reeve 1952). We then dissected the reproductive
271 tract into Grace's Insect Medium. For females, we separated the seminal receptacle (SR) and a
272 randomly chosen spermatheca (ST) from the rest of the reproductive tract and transferred them to
273 a slide. For males, a randomly chosen testis was separated from the rest of the reproductive tract

274 and transferred to a slide. In both cases, we then applied a cover slip and took images under an
275 EVOS FL digital inverted microscope. Using ImageJ, we measured the length of the SR, the
276 height and width of the ST, and the length of the testis, from these images. Measurements were
277 replicated across five individuals per genotype.
278
279 *2.6.2 Sperm length*
280 We dissected sperm out of the seminal vesicles of reproductively mature virgin males into
281 Grace's insect medium on a slide (N=5 replicates per genotype). Sperm were gently teased apart
282 with a probe when necessary. The slides were stained with SpermBlue (Microptic), and imaged
283 under an EVOS FL digital inverted microscope. In ImageJ, we measure sperm length for 5-10
284 randomly chosen sperm per individual.
285
286 *2.6.3 Body mass and reproductive tract mass*
287 We collected both female and male virgins and aged them to reproductive maturity (N=5
288 replicates per sex, per genotype). We measured body and reproductive tract mass according to
289 Holman et al. (2008). Flies were dissected in distilled water onto a piece of preweighed foil. For
290 females, the reproductive tract (including the bursa, SR, ST, parovaria, and ovaries) was removed
291 and transferred to another piece of pre-weighed foil. For males, the reproductive tract (including
292 testes, seminal vesicles, accessory glands, and the ejaculatory bulb) was removed and transferred
293 to another piece of pre-weighed foil. For both males and females, the foils holding the body and
294 reproductive systems of the flies were then placed in a drying oven at 60°C for 18 ± 1 h and
295 weighed on a Mettler Toledo UMX2 balance to the nearest 0.1 μg.
296

297 *2.7 Second-male courtship behavior assay*

298 To identify variation in male courtship across genotypes, and to determine whether our
299 experimental manipulation affects male courtship behavior, we recorded male courtship for each
300 cross type on video. We recorded videos using a modified FlyPi setup, which combines a
301 Raspberry Pi (Raspberry Pi Foundation, Cambridge, UK), pi camera, and 3D printed parts (Maia
302 Chagas et al. 2017). Crosses were set up in a modified cell culture plate consisting of six culture
303 wells, each with a 3 cm diameter and a small amount of cornmeal media in the bottom, allowing
304 us to record six crosses simultaneously. Male and female flies were aspirated without
305 anesthetization into each cell culture well, and the plate was videotaped for two hours. The full
306 videos were scored for successful copulations, latency to copulation, and copulation duration.
307 The first 30 minutes of each video was also scored in more detail for the frequency of courtship
308 song, male tapping, number of male licking attempts, and copulation attempts. Recorded matings
309 were set up until we had five replicate recordings per cross type.

310

311 *2.8 Statistical analyses*

312 All statistical analysis and figure construction in this study was performed with R version
313 4.3.1. All linear models were run using deviation-coded contrasts. This does not change the fit of
314 the model, but affects the interpretation of regression coefficients such that the intercept
315 corresponds to the grand mean and main effect estimates represent deviations from the mean.
316 Model estimates are therefore robust to whichever line is used as the baseline in each
317 comparison, which is preferable in our experiments where none of our genotypes were *a priori*
318 designated as the baseline level.

319

2.8.1 CHC composition

We assessed variation in CHC composition using a principal components analysis (PCA), which we computed using the prcomp function in R. To test for significant differences among our principal components, we performed a multivariate analysis of variance (MANOVA) using the manova function.

2.8.2 Postmating reproductive trait morphology

We assessed variation in female and male reproductive morphology by fitting linear models using the lm function in R. To test whether male or female genotype predicts the variation we observed in reproductive morphology, we ran linear models for each morphological trait separately as the dependent variable. Male or female genotype was always included as the predictor. The GFP-labelled *D. melanogaster* line was incorporated into each model as a baseline.

2.8.3 Second-male courtship effort, female second-mating frequency, and second-male paternity

We assessed variation in second-male courtship by fitting a linear model using the lm function in R. Second male courtship effort was the dependent variable, with male genotype, female genotype, their interaction, and male perfuming treatment, as predictors.

We assessed variation in female second-mating frequency by fitting a binomial generalized linear model using the glm function in R. We coded our response as a binomial variable, with one column representing the number of successful second-matings, and the other column representing the number of failed second-matings for each cross type (N=32 total cross types). The generalized linear model included male genotype, female genotype, and male

343 perfuming treatment as predictors (this dataset did not allow us sufficient power to assess
344 interaction terms).
345 Variation in second male siring success was also assessed with a binomial generalized
346 linear model. We coded our response as a binomial variable, with one column representing the
347 number of offspring sired by the second male ("successes"), and the other column representing
348 the number of offspring sired by the first male ("failures") for each cross (N=196 total crosses).
349 Our generalized linear model included male genotype, female genotype, the interaction between
350 male and female genotype, male perfuming treatment, and the interaction between female
351 genotype and male perfuming treatment as predictors. California males, California females, and
352 conspecific-perfumed males were always incorporated into the models as baselines.
353 To assess the overall significance of our model components, we computed type III
354 analyses of variance (ANOVAs) using the same models, with the aov function in R. Effect sizes
355 (and 95% confidence intervals) of our model components were estimated using the resi function
356 in R, from the RESI package (Vandekar et al. 2020).
357
358 *2.8.4 Correlations among datasets*
359 Finally, we looked for correlations among our datasets. Across all pairwise genotype
360 combinations we assessed the strength of association between: second-male courtship effort and
361 second-male mating success; second male courtship effort and paternity share (P2); female
362 readiness to remate and second-male paternity share (P2); and, male and female reproductive
363 trait variation and second-male paternity share (P2). To do this, in each case, we computed
364 Spearman's rank correlation coefficients using the cor.test function in R. Each correlation was

assessed separately for experiments involving conspecific-perfumed males and heterospecific-perfumed males.

3 Results

3.1 Males differ in CHC composition based on genotype and perfuming status

We quantified CHC composition across male genotypes and perfuming treatments to characterize the variation present across lines, as well as to verify that our perfuming treatment was effective. We found that CHC composition varied substantially across male genotypes ((Figure 2a,b, Table S1, Table S2). For each of the first three PCs—which together account for 92.3% of the variation observed—we detected a significant genotype effect (MANOVA $p < 0.001$; Table S1). CHC composition was also shifted by our perfuming treatment, as shown by significant treatment effects for PC3 ($p = 0.004$) and PC4 ($p < 0.001$) (MANOVA; Figure 2b, Table S1). These two PCs account for 8.6% of the variation in CHC composition.

3.2 Postmating reproductive traits differ more among males than among females

We quantified variation in male and female traits, focusing on postmating reproductive traits, to assess whether these might influence patterns of sperm use. We found that all measured male traits differed among genotypes, including two overall size traits and three reproductive size traits (Figure 3, Table S3). Differences among female lines were more modest and only observed for overall size and one reproductive size trait, RT mass (Figure 3, Table S3).

In terms of non-reproductive traits, both male and female Taiwan individuals consistently had shorter thoraxes but also the largest dry non-RT body mass. Other differences among lines

387 were sex-specific (for example, in Greece males and Malaysia females) and often more modest
388 (Table S3).
389 For reproductive traits, California females exhibited the highest RT dry mass and Taiwan
390 females exhibited the lowest; however, female lines did not differ for either SR length or ST area
391 (Figure S1). In contrast, male lines differed in RT mass (Taiwan males had lower RT mass, while
392 Greece males had higher RT mass, than average), as well as testis length and sperm length
393 (Figure 3, Figure S1). California and Greece males had longer testes than average, while
394 Malaysia and Taiwan males had shorter testes. Interestingly, California and Taiwan males had
395 longer sperm than all other lines, and Malaysia and Greece males had significantly shorter
396 sperm. GFP sperm length was intermediate. This indicates that sperm length and testis length are
397 not closely associated among our *D. melanogaster* lines: Taiwan males had short testes but also
398 had among the longest sperm, while the inverse pattern was observed for Greece males.
399
400 *3.3 Courtship effort varies consistently among male genotypes, but not among female genotypes*
401 *being courted or with perfuming*
402 We evaluated courtship across each male-female genotype combination to better
403 understand if and/or how premating processes influence cryptic female choices, including via our
404 perfuming treatments. We measured courtship effort as the number of courtship behaviors
405 exhibited per minute until copulation occurred or until our observation period ended (at two
406 hours). Note that only 9 of 162 scored pairings resulted in copulation within 2 hours. Courtship
407 intensity varied significantly among male genotypes (Table 1), with Greece and Taiwan males
408 courting significantly less than the others (Figure 4, Table S4). Male courtship intensity was also
409 marginally reduced in heterospecific-perfumed males, but this effect was not statistically

410 significant (Figure 4, Table S4). In addition, although female line identity had only a marginal
411 influence on courtship (Table 1), we did find that Taiwan females were consistently courted less
412 than average across male genotypes (Figure 4, Table S4). We did not detect any significant
413 female-by-male genotype interactions. Consistent with these observations, the estimated effect
414 size of male genotype on male courtship effort was more than 2.5 times that of any other effect in
415 our model (Table 1).
416
417 *3.4 Readiness to remate differs among female genotypes and depends on male genotype, but not*
418 *on male perfuming status*
419 For each cross type, we assessed whether females chose to mate with second males
420 versus refused the second-male mating. Remating was evidenced by whether a female produced
421 offspring from the second male. We found that female genotypes varied in their readiness to
422 remate following a first mating with the tester male (Table 2): California females remated more
423 readily while Greece and Taiwan females mated less readily than average (Figure 5, Table S5).
424 Male genotype also affected remating (Table 2): California males were more consistently
425 successful in these second matings, while Greece males were less successful on average (Figure
426 5, Table S5). In contrast, we detected no effect of male perfuming status on second-male
427 premating success (Figure 5, Table 2, Table S5).
428
429 *3.5 Female genotypes vary in their overall use of sperm from the second male, and in whether*
430 *they adjust sperm use according to second-male perfuming status*
431 We assessed how paternity of second-males (P2) was affected by genotype and by our
432 perfuming manipulation. We found strong and significant differences in second-male paternity

depending on both female and second-male genotype, perfuming treatment, female by male genotype interaction effects, and female by treatment interaction effects (Table 3). The largest of these effects was male genotype, while the smallest was the female by treatment effect; the estimated effect size of second male genotype was ~2 times that of female genotype, and ~3.5 times that of the male by female interaction (Table 3). When evaluated with a complete linear model across all genotype pairs, we detected several general genotype effects. In particular, Taiwan males gained higher P2 than average, while Greece and Malaysia males had reduced paternity share (Figure 6, Table 4). In comparison, Greece females exhibited a higher P2 than average, while Taiwan females exhibited a lower P2 than average, across all genotype pairs (Figure 6, Table 4). Despite these general effects, second-male paternity share also varied substantially depending on the particular male-female combination. For instance, Greece females exhibit lower average P2 when paired with Greece second males, while Taiwan females increase P2 when paired with Greece second males (Figure 6, Table 4).

We also found that females adjust sperm use according to male perfuming treatment, including in female genotype-specific ways. Compared to conspecific-perfumed males, heterospecific-perfumed males showed a modest but significantly higher second male paternity overall (Figure 6, Table 4), but female genotypes vary in whether and how sperm use patterns were affected by perfuming (Figure 6, Table 4). Specifically, Taiwan females show an overall reduction in paternity associated with male heterospecific perfuming status, while Greece and Malaysia females exhibit slight overall increases in P2 associated with male heterospecific perfuming (Figure 6, Table 4). In comparison, California females do not appear to respond to male CHC perfuming when making sperm use choices.

3.6 Both pre- and postmating choices can be affected by premating behavior, but sperm length shows the most consistent association with patterns of preferential sperm use

We next assessed correlations between datasets to better understand how these variables might influence one another. First, we evaluated the relationship between second-male courtship effort and second-male mating success—i.e., is premating courtship intensity associated with premating success? We did not see a consistent association for either conspecific-perfumed males (Figure S3a; Spearman's rank correlation $\rho = 0.308$, $p = 0.246$) or heterospecific-perfumed males (Figure S3b; Spearman's rank correlation $\rho = 0.174$, $p = 0.519$). Instead, readiness to remate seems to be more clearly associated with individual female line differences. For instance, California females tend to remate most readily, and Greece and Taiwan least readily, regardless of the courtship intensity of the male line they are paired with (Figure S3a,b).

Second, we assessed the relationship between second male courtship effort and paternity share (P2)—i.e., do females use premating signals to make postmating decisions? We found that when males are perfumed by conspecifics, male courtship effort is significantly correlated with siring success (Figure 7a; Spearman's rank correlation $\rho = 0.535$, $p = 0.035$). When males are perfumed by heterospecifics, the correlation is positive but not significant (Figure 7b; Spearman's rank correlation $\rho = 0.353$, $p = 0.180$). This weak positive trend between courtship effort and P2 appears to be largely driven by California males, which are the most active courters and also received the greatest share of paternity (Figure 7a,b), an association which is unclear in the other male lines. Thus, we infer that the relationship between these variables is likely influenced by additional factors not measured here; for instance, Greece males, which have low siring success, have been shown to exhibit especially long interpulse intervals during courtship

478 song, which are less preferred by *D. melanogaster* females (Pischedda et al. 2014a, Kyriacou and
479 Hall 1982).

480 We next assessed the relationship between female readiness to remate and second-male
481 paternity share—i.e., is premating choosiness associated with postmating choosiness? We found
482 no correlation among these factors for conspecific-perfumed males (Figure 7c; Spearman's rank
483 correlation $\rho = -0.095$, $p = 0.726$), and a slight association for experiments with heterospecific-
484 perfumed males (Figure 7d; Spearman's rank correlation $\rho = 0.502$, $p = 0.048$). These weak or
485 absent associations are consistent with heterogenous effects we observe among female lines. For
486 instance, while Greece females showed relatively high premating choosiness (remating in only
487 59% of trials) and the highest average P2 of any genotype (consistent with strong second male
488 preference), Taiwan females exhibit a similar premating choosiness (60% remating frequency)
489 but significantly lower average P2 (indicating no consistent postmating preference for first or
490 second male sperm) (Figure 7c,d). This relationship therefore appears to vary substantially
491 depending upon which of the four genotypes is considered.

492 Finally, we examined the relationship between second-male paternity share and
493 reproductive trait variation among lines. Of RT mass (both male and female), SR length, testis
494 length, sperm length, and SR length minus sperm length, only sperm length and MRT mass were
495 significantly associated with P2. Of these two, MRT mass was significantly negatively correlated
496 with P2 among heterospecific-perfumed males (Figure S3d; Spearman's rank correlation $\rho = -$
497 0.679, $p = 0.004$), but not among conspecific-perfumed males (Figure S3c; Spearman's rank
498 correlation $\rho = -0.449$, $p = 0.081$). These relationships appear to be substantially influenced by
499 Taiwan males, which have high P2 but comparatively low RT mass. In comparison, the
500 relationship between sperm length and P2 was consistently and strongly positive for both

conspecific-perfumed (Figure 7e; Spearman's rank correlation $\rho = 0.631$, $p = 0.009$) and heterospecific-perfumed groups (Figure 7f; Spearman's rank correlation $\rho = 0.728$, $p = 0.001$). Second males with longer sperm experience greater siring success. Specifically, when the second male's sperm is longer than the tester male (as for California or Taiwan second males), P2 is generally higher; whereas when the second male sperm is shorter than the tester male (as for Malaysia and Greece), P2 is generally lower. Together, these data indicate that females preferentially use longer sperm—a generic preference that appears as last male precedence when the second male's sperm is longer than the first (tester) male's sperm, but can override last male precedence when the second male's sperm is relatively shorter.

4 Discussion

In this study, we sought to understand whether and how females make plastic choices about sperm use. We found that sperm use patterns vary based on male genotype, female genotype, their interaction, as well as based on male pheromone profile (even when male identity is the same). Among these effects, male sperm length, specifically the relative difference in sperm length between the first and second male, appears to play a particularly important role in determining female sperm use patterns. While sperm length has been recognized as an important component of male-male competition (e.g., Snook 2005, Immler et al. 2011, Lüpold and Pitnick 2018), our analysis enables us to infer that the preference for longer sperm is also female-mediated, context-dependent, and controlled by factors other than the size of the female sperm storage organs, as we expand upon below.

523 *4.1 Female sperm use choices vary among lines in their direction and magnitude, and in the*
524 *criteria used to evaluate males*

525 Consistent with previous work in *D. melanogaster*, we observe clear evidence for sperm
526 choice mediated by females (Clark and Begun 1998, Clark et al. 1999, Miller and Pitnick 2002,
527 Chow et al. 2010). Female lines show consistent patterns of sperm use in control crosses (with
528 their own unmanipulated second males), and significant shifts in these patterns in response to
529 changing either second-male genotype or perfuming treatment or both (depending on the female
530 line). This result underscores the potential for this female-mediated process to influence
531 genotypic representation among the resulting offspring, and to generate sexual selection on males
532 (Pitnick et al. 2001).

533 We found substantial and consistent variation among our four female lines in the
534 direction and magnitude of preferential sperm use patterns. *D. melanogaster* is conventionally
535 described as having second (or last) male sperm precedence (Lefevre and Jonsson 1962,
536 Pischedda and Rice 2012). However, depending on the specific male-female combination, our
537 estimated preference for the sperm of second-males ranged from 0.2 to 0.95. Values below 0.5
538 (e.g., Taiwan females with Malaysia males) indicate consistent first-male precedence in these
539 specific male-female combinations. Moreover, female lines differ in how strongly they
540 differentiate among sperm donors. For example, with the exception of California males (which
541 they strongly prefer; average P2 = 0.79), California females do not consistently differentiate
542 between the first and second males' sperm when paired with the other three second male lines in
543 our experiment (i.e., mean P2 is approximately 0.5, with a broad range of values among
544 individual CA females; Figure 6, Table S6).

We also clearly observed among-line variation in the characteristics that females use to evaluate males as reproductive donors. In particular, Greece females (and to some extent Taiwan and Malaysia females) modulate their use of second male sperm in response to perfuming status, albeit in complex ways. For example, Greece females decrease their use of Greece sperm when these males are heterospecific-perfumed, but Taiwan females increase their use of Greece sperm after the same treatment (Figure 6, Table S7). In comparison, male perfuming appears to have no influence on cryptic choice by California females. This diversity in sperm choice patterns depending on intrinsic (female genotype) and extrinsic (perfumed status) factors points to a much greater heterogeneity of sperm use decision-making among *D. melanogaster* females than simple last male precedence (see also Laturney et al. 2018). Given the broad geographic origin of these lines, differences in the magnitude and direction of female choices could reflect reproductive or other differences among lines that operated prior to their collection and lab establishment.

4.2 Cryptic female choice is strongly shaped by male traits, especially sperm length

Within the diversity of individual sperm use criteria, however, we did observe several broad patterns. In particular, every female line differentiated among male genotypes in at least one postmating experimental context, indicating that one or more aspects of male genotype are important in making paternity allocation decisions. Consistent with this, male genotype had the largest estimated effect size on second male paternity share (P2; Table 3), nearly twofold greater than the next largest effect (female genotype). Our analysis also identifies specific male traits that likely mediate these male genotype effects. As noted above, some female lines adjust sperm use in response to perfuming treatment, indicating that male cuticular hydrocarbon profile affects

568 their postmating choices. Among conspecific-perfumed second males, paternity share is also
569 associated with courtship effort suggesting that females may prefer the sperm of male genotypes
570 who court more, although this effect is shaped largely by California males. Most strikingly,
571 however—and more consistently than either chemosensory or courtship traits—we find that
572 sperm length is positively associated with preferential sperm use across many contexts and
573 conditions. The majority of female lines preferentially used the second-male's sperm when that
574 sperm was longer than the first-male's sperm (i.e., when California or Taiwan was the second
575 male). Moreover, in several cases (for example, when Taiwan females mate with Malaysia
576 males—see Figure 6) female lines subverted the expected precedence for last males' sperm
577 specifically when the first (GFP tester) male had longer sperm than the second male (i.e. Greece
578 and Malaysia males). That is, our results indicate that 'long-sperm precedence', in addition to
579 'last-male sperm precedence' (Lefevre and Jonsson 1962), jointly contribute to female sperm use
580 patterns in *D. melanogaster*.

581 Finally, although our data indicates diverse criteria for female sperm choice overlaid on a
582 generic preference for longer sperm, it also indicates that these patterns are not explained by at
583 least one studied mechanism of female sperm choice—SR length (Miller and Pitnick 2002). SR
584 length has been proposed as a primary mechanism via which females discriminate between
585 sperm of different lengths (Pitnick et al. 1999); in particular, females with longer SRs are thought
586 to be most selective because they can better discriminate between the sperm length of the first
587 and second male. However, while we observe clear variation in female discrimination of sperm
588 based on length—e.g., Greece females exhibit generally high P2, regardless of length, whereas
589 California females only exhibit high P2 for their own (long sperm) males (Figure 6, Table 4)—
590 we do not observe significant variation in SR length among our female lines (Figure 3, Table S3,

591 Table S2). Thus, SR length is not the mechanism by which these diverse *D. melanogaster* female
592 lines discriminate against short sperm in our experiments.
593
594 *4.3 Evolutionary implications of the observed variation in female sperm choice*
595 Consistent patterns in choice criteria among female genotypes provide an opportunity to
596 evaluate the nature of past drivers of female choice in our system, including hypotheses where
597 cryptic female choice is not beneficial to females versus where it has specific fitness benefits.
598 For example, one proposal is that female sperm use patterns result primarily from manipulation
599 by males; that is, cryptic female choice is the product of sexual conflict rather than providing an
600 adaptive benefit to females. In this case, female sperm use patterns are expected to be random
601 with respect to actual measures of male quality. Our data do not convincingly support this
602 hypothesis, given the general postmating female preferences we observe for males who have the
603 longest sperm and, under some conditions, for males that court frequently.
604 Alternatively, Birkhead (1998) proposed that female sperm choice might function to bias
605 sperm use against the sperm of closely related males. However, our findings are not consistent
606 with inbreeding avoidance as a primary function of cryptic female choice. If that were the case,
607 second-male success (against the unrelated tester first male) should consistently be lower in
608 crosses with males from the same line as females, compared to males from different lines.
609 However, female lines differed in their choice of own male sperm, ranging from consistent high
610 preference to low preference.
611 Apart from inbreeding avoidance, other hypothesized functions of sperm choice include
612 female benefits from sire discrimination (e.g., choice of high-quality males [‘good genes’
613 hypothesis; Eberhard 1996], or choice of attractive males [‘sexy sons’ hypothesis; Weatherhead

614 and Robertson 1979]). We find that females have a generic preference for longer sperm (Figure
615 7e,f); in this case, choosing longer-spermed males increases the frequency of longer-spermed
616 (i.e. 'sexier') sons, which themselves could benefit from the same generic preference. As studies
617 have shown that long sperm have a competitive advantage in displacing shorter sperm (Lüpold et
618 al. 2012), this preference likely also aligns specifically with higher reproductive fitness in males.
619 Thus, our data support the hypothesis that females could gain indirect fitness benefits from
620 exercising cryptic choice based on specific male traits like sperm length.

621 The differences we observe among female lines in the criteria they used for sperm choice
622 also have implications for the expected future drivers of postmating reproductive evolution.
623 Some of our lines altered sperm use patterns based on contact pheromones (especially Greece,
624 but also Taiwan and Malaysia), indicating that pheromone bouquet can be a criterion for
625 evaluating preferred sires. Other lines (e.g., California) were insensitive to pheromone
626 manipulation in their sperm use choices. Variation in choice criteria makes it more challenging
627 for males to tailor specific traits to satisfy, or manipulate (Ryan 1990), a broad range of female
628 biases. Moreover, when the specific preferences of females differ according to male trait values
629 and male trait identity, there is no single most preferred male genotype, and variation should be
630 maintained at male traits. This expectation is consistent with our observation, and with previous
631 analyses (e.g., Clark et al. 1999, Miller and Pitnick 2002, Chow et al. 2010), showing that the
632 outcomes of cryptic female choice are demonstrably influenced by male by female interactions.

633 That these criteria for female sperm choice can also vary behaviorally is particularly
634 evident in the case of Greece females. When sequentially mated with males from other lines,
635 Greece females uniformly favor whichever genotype (tester versus second male) has the longer
636 sperm. However, when mated with their own conspecific-perfumed second males, Greece

637 females preferentially use this sperm (Figure 6), even though it is significantly shorter than the
638 first (tester) male (Figure 3). In this case, the female preference for local males appears to
639 override their generic preference for longer sperm. We know that this decision is both female-
640 mediated and based on chemosensory assessment, because it is reversed when Greece females
641 are paired with heterospecific-perfumed Greece males. Greece females instead favor the longer
642 (tester male) sperm in these pairings (Figure 6), even though these Greece males are identical in
643 every sense to conspecific-perfumed males—except for their CHC profile. Flexible changes in
644 preference, such as this case where females specifically prefer own males despite generic
645 preferences for longer sperm, is another potential mechanism by which sexually selected
646 variation could be maintained in males.
647

648 **5 Conclusion**

649 The direction and magnitude of female-mediated sperm choice, as well as the male traits
650 influencing these choices, varies significantly among four female lines of *D. melanogaster*. This
651 diversity in female sperm choices is overlaid on a generic preference for longer sperm,
652 suggesting that paternity decisions may ultimately benefit female fitness indirectly through the
653 production of higher-quality offspring or more attractive offspring. Interestingly, at least one
654 proposed proximate mechanism of female sperm choice—SR length—does not account for
655 variation in observed sperm use. Diversity in cryptic female choices—both in the specific
656 modalities used to assess and choose among males, and in the plasticity of these choices—may
657 explain how variation in male postmating sexually selected traits is maintained over time.
658

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Table 1. Factors affecting male courtship effort. Overall significance of model components and their effect sizes in predicting courtship effort as assessed using ANOVAs.

Model component	Sum Sq	Df	*F*	*p*	Effect size	95% CI
Female line	1.397	3	2.140	0.098	0.087	0.000-0.443
Second male line	7.349	3	11.254	<0.001 ***	0.392	0.275-0.897
Treatment	0.824	1	3.786	0.054	0.120	0.000-0.301
Female line x second male line	3.663	9	1.870	0.061	0.226	0.153-0.569

835

Table 2. Factors affecting female remating frequency. Overall significance of model components and their effect sizes in predicting female remating as assessed using ANOVAs.

Model component	LR Chi sq	Df	*p*	Effect size	95% CI
Female line	19.776	3	<0.001 ***	0.707	0.199-1.329
Second male line	13.787	3	0.003 **	0.439	0.000-1.263
Treatment	0.061	1	0.805	0.000	0.000-0.323

836

Table 3. Factors affecting second male paternity share (P2). Overall significance of model components and their effect sizes in predicting female use of second male sperm (P2) as assessed using ANOVAs.

Model component	LR Chi sq	Df	*p*	Effect size	95% CI
Female line	448.71	3	<0.001 ***	0.331	0.221-0.616
Second male line	1327.93	3	<0.001 ***	0.632	0.492-0.980
Treatment	37.91	1	<0.001 ***	0.063	0.000-0.218
Female line x second male line	297.72	9	<0.001 ***	0.170	0.020-0.507
Female line x treatment	21.79	3	<0.001 ***	0.000	0.000-0.225

837

Table 4. Specific genotype, treatment, and interaction effects on second male paternity share (P2). Shown is the binomial generalized linear model of variation in second male paternity share (P2), depending on female line, second male line, treatment group, the interaction between female line and second male line, and the interaction between female line and treatment group. California females, California males, and conspecific-perfumed males are incorporated into the model as a baseline.

Model component	Estimate	Std. error	*z*	*p*
Intercept	0.723	0.023	30.860	<0.001 ***
Female line (Greece)	0.889	0.051	17.372	<0.001 ***
Female line (Malaysia)	0.024	0.032	0.733	0.463
Female line (Taiwan)	-0.385	0.036	-10.842	<0.001 ***
Second male line (Greece)	-0.616	0.039	-15.688	<0.001 ***

Second male line (Malaysia)	-0.921	0.035	-26.074	<0.001 ***
Second male line (Taiwan)	0.446	0.039	11.496	<0.001 ***
Treatment (het-perfumed)	0.132	0.022	6.144	<0.001 ***
Female line (Greece) x second male line (Greece)	-0.341	0.087	-3.904	<0.001 ***
Female line (Malaysia) x second male line (Greece)	-0.012	0.053	-0.230	0.818
Female line (Taiwan) x second male line (Greece)	0.265	0.057	4.665	<0.001 ***
Female line (Greece) x second male line (Malaysia)	-0.083	0.068	-1.211	0.226
Female line (Malaysia) x second male line (Malaysia)	-0.081	0.049	-1.661	0.097
Female line (Taiwan) x second male line (Malaysia)	-0.592	0.058	-10.247	<0.001 ***
Female line (Greece) x second male line (Taiwan)	0.792	0.087	9.126	<0.001 ***
Female line (Malaysia) x second male line (Taiwan)	0.033	0.053	0.628	0.530
Female line (Taiwan) x second male line (Taiwan)	-0.028	0.058	-0.481	0.630
Female line (Greece) x treatment (het-perfumed)	0.098	0.044	2.205	0.027 *
Female line (Malaysia) x treatment (het-perfumed)	0.073	0.030	2.435	0.015 *
Female line (Taiwan) x treatment (het-perfumed)	-0.123	0.034	-3.658	<0.001 ***

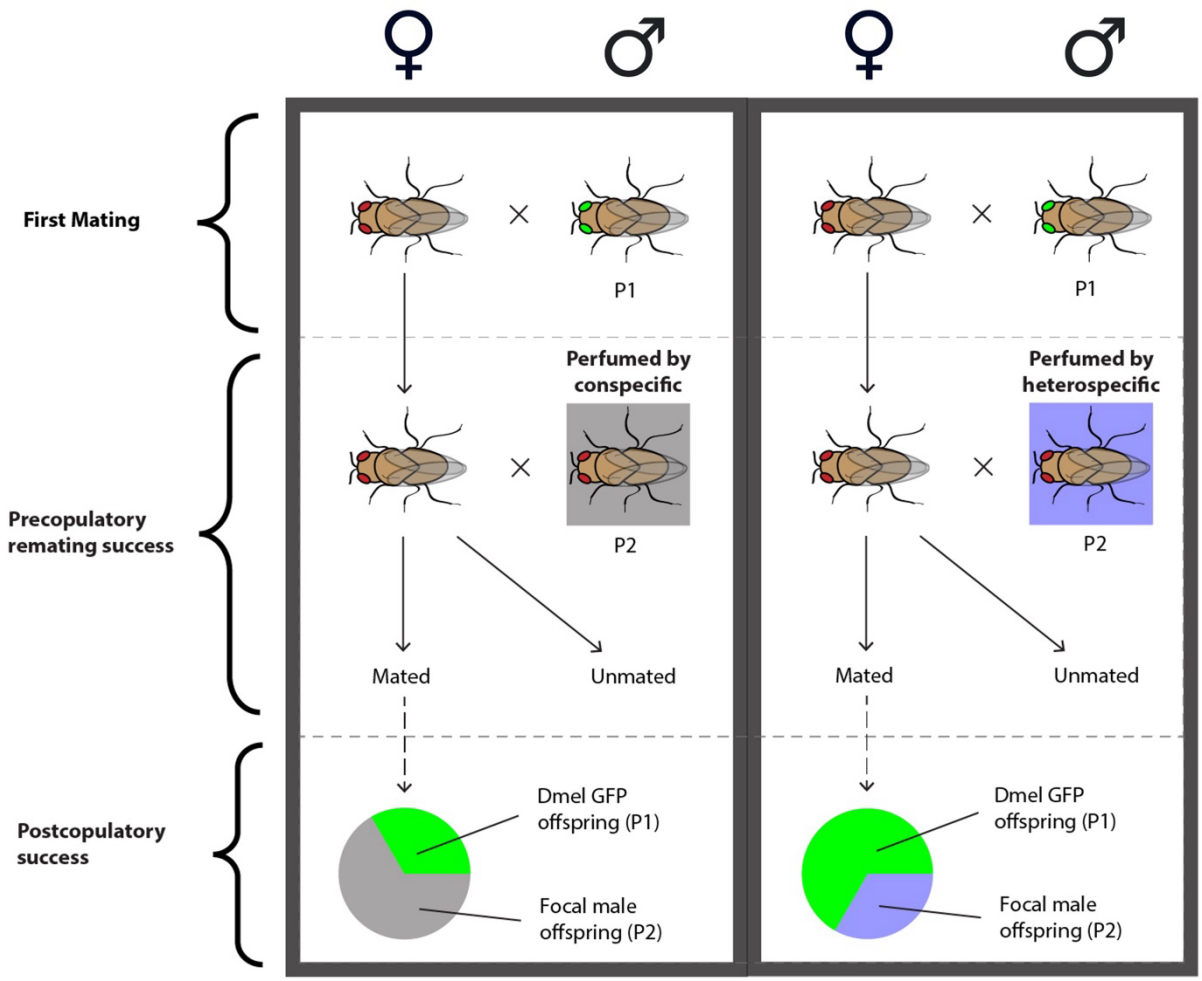

Figure 1. Experimental procedure for assessing paternity share of second (focal) males. Females were first mated with a GFP tester male. After 48 hours, these females were paired for second matings with focal males, which were either perfumed by conspecifics (left, control) or by heterospecifics (right, treatment). Unmated females were removed from the experiment. In remated females, we assessed paternity for first and second males based on the presence/absence of the GFP marker in resulting offspring.

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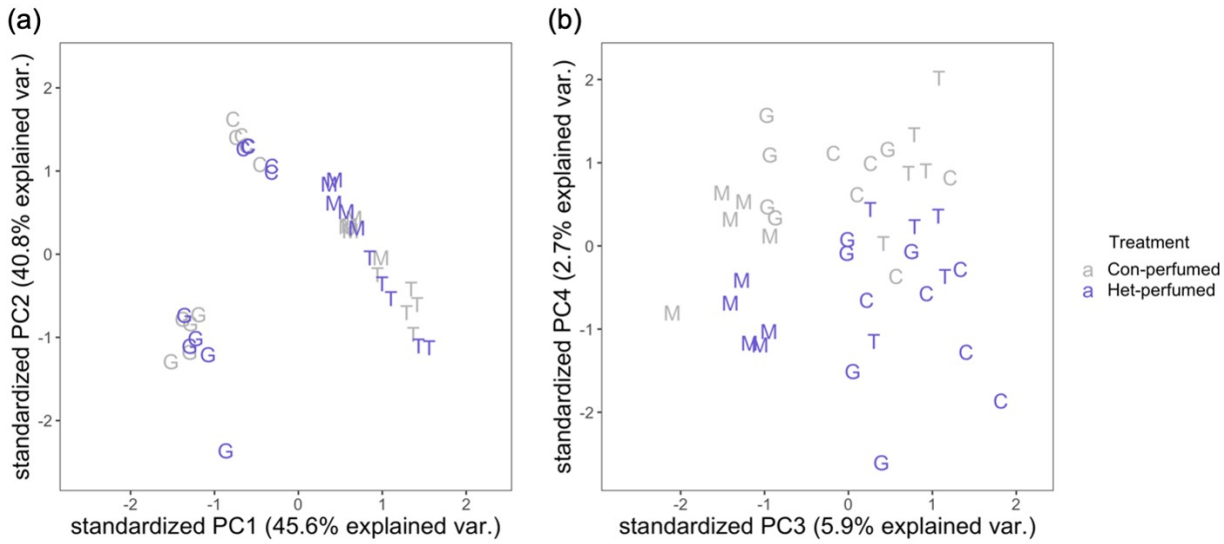

Figure 2 CHC composition varied based on genotype (G, Greece; T, Taiwan; C, California; and M, Malaysia) and perfuming status (conspecific-perfumed males shown in grey, heterospecific-perfumed males shown in purple) (Table S1). Both panels show standardized principal component (PC) values generated from multivariate CHC composition data for replicate males of each genotype and treatment. Left panel: PCs 1 and 2 largely reflect differences between male genotypes. Right panel: PCs 3 and 4 reflect additional shifts in CHC composition associated with heterospecific perfuming treatment.

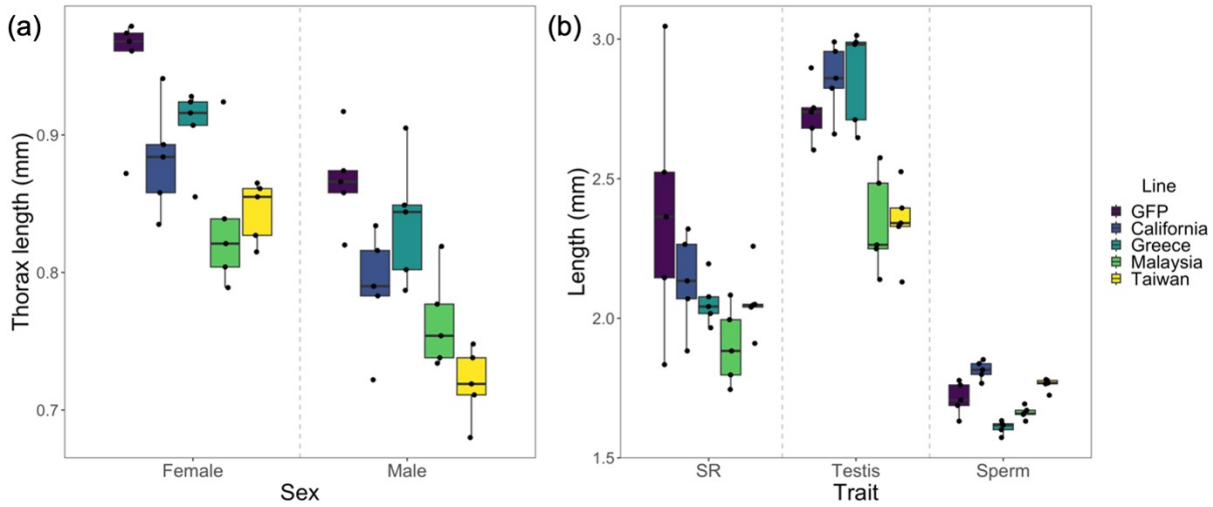

Figure 3. Variation in (a) thorax length in mm and (b) reproductive trait length in mm in females and males across *D. melanogaster* lines. We observed significant variation among lines for thorax length (both males and females), testis length, and sperm length, but SR length did not significantly differ between lines (Table S3). Box plots show the median (central line)

and first and third quartiles; whiskers extend to the smallest and largest values (not including outliers >1.5x the inter-quartile range).

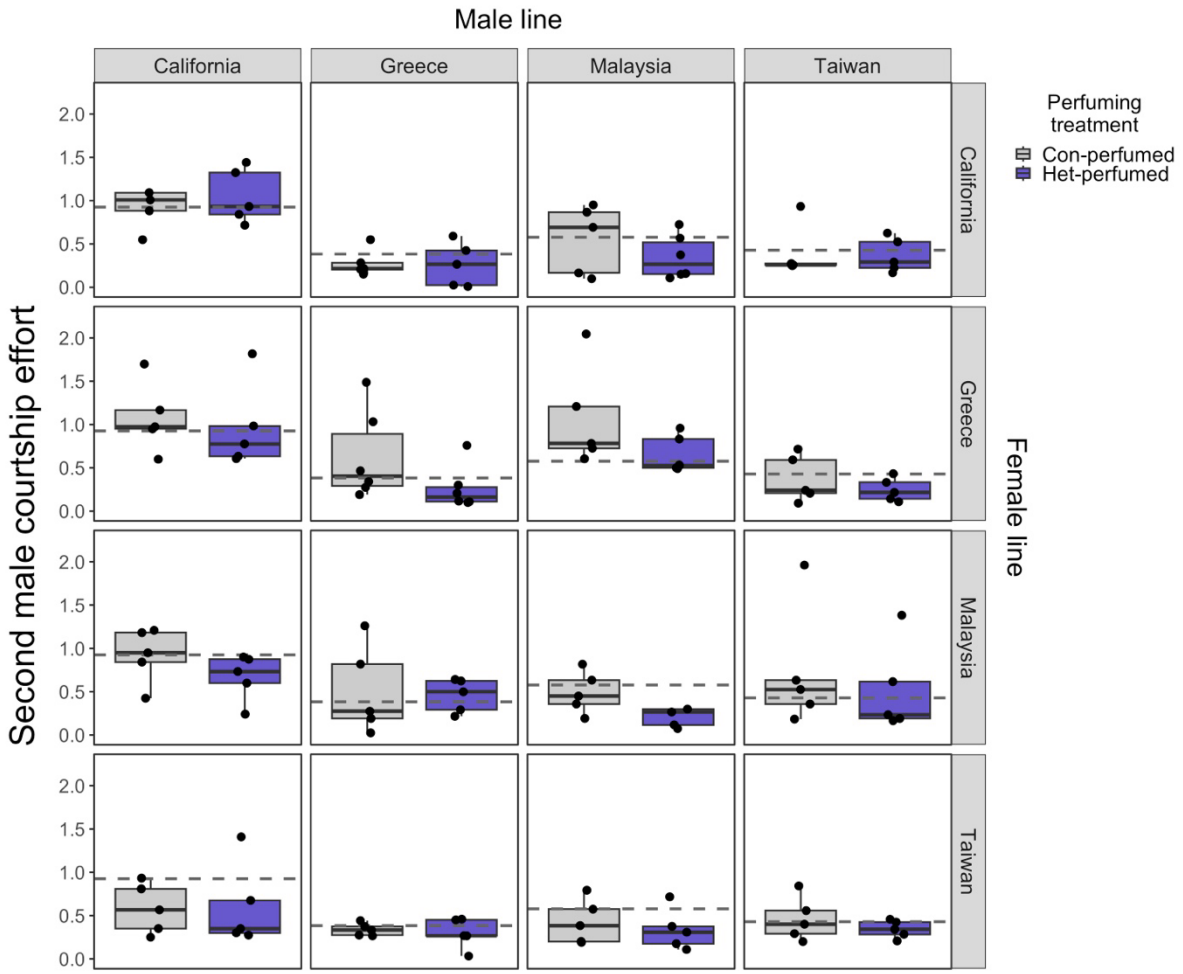

Figure 4. Courtship effort (courtship behaviors per minute) consistently differed between second male genotypes but did not consistently vary by the female genotype being courted or by male perfuming status (Table 1, Table S4). Conspecific-perfumed males are shown in grey, and heterospecific-perfumed males are shown in purple. The dashed line indicates the average courtship effort for each male line. Box plots show the median (central line) and first and third quartiles; whiskers extend to the smallest and largest values (not including outliers >1.5x the inter-quartile range). The y-axis has been truncated to make variation in the data easier to visualize; the non-truncated data (as used in statistical analyses) is shown in Figure S2.

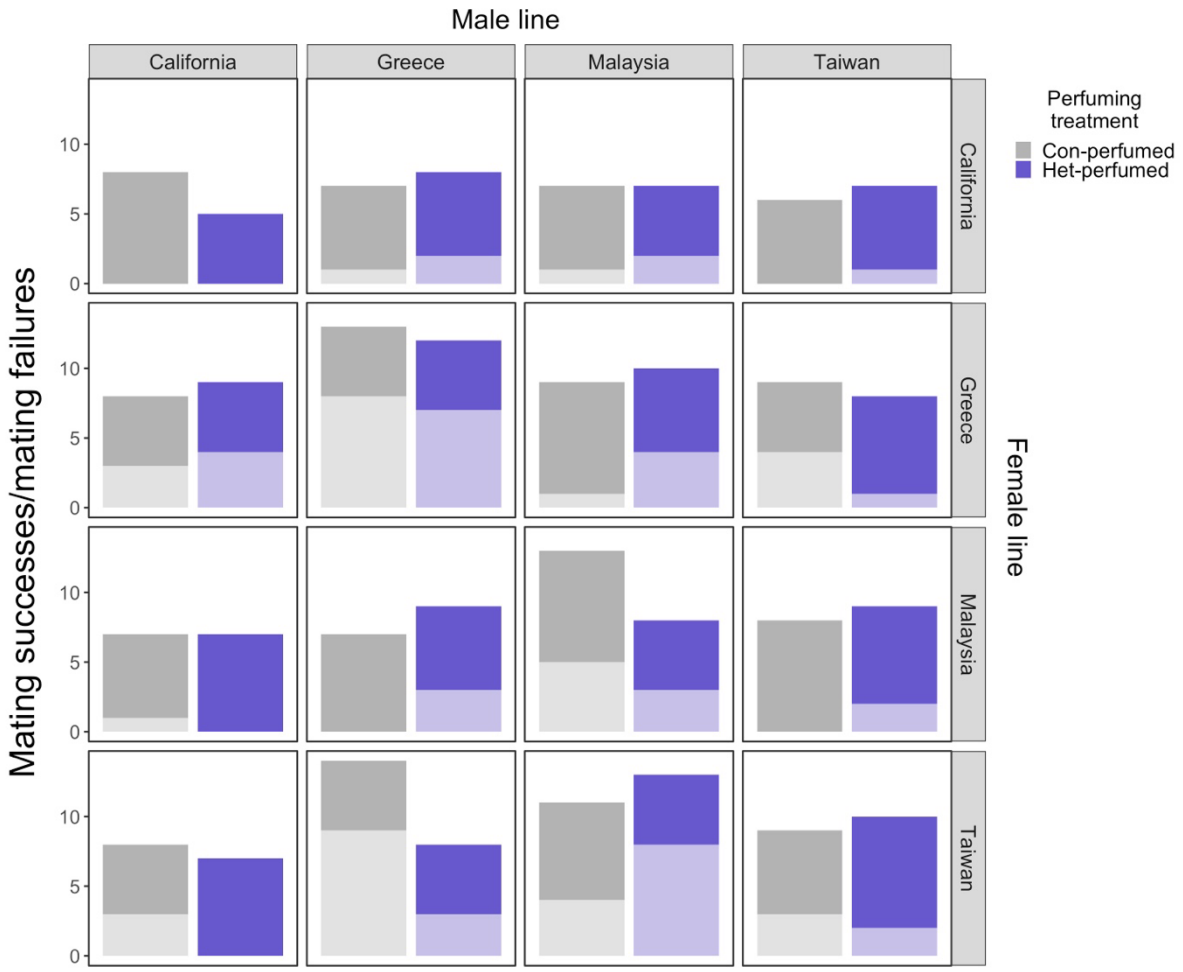

Figure 5. Counts of mating successes (dark shading) and mating failures (light shading) differs across male (column) and female (row) genotypes (Table 2, Table S5). Results are shown separately for conspecific-perfumed males (grey) and heterospecific-perfumed males (purple) within each male x female panel.

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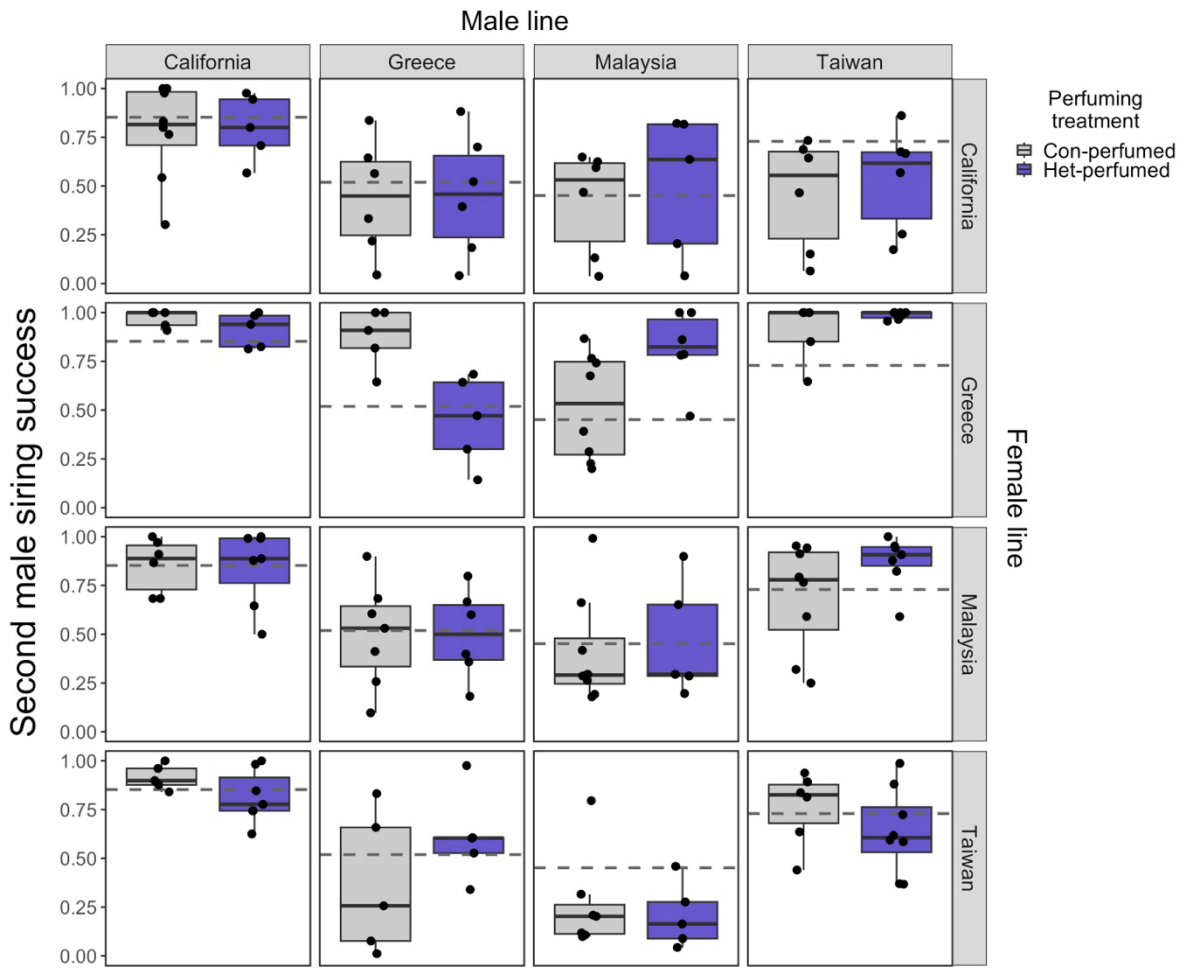

Figure 6. Female use of second male sperm (P2) varies across female genotypes, male genotypes, and female by male genotype combinations (Table 3, Table 4). P2 also varies depending on male perfuming status, as shown in grey (conspecific-perfumed males) and purple (heterospecific-perfumed males). The dashed line indicates the average P2 for each male line. Box plots show the median (central line) and first and third quartiles; whiskers extend to the smallest and largest values (not including outliers >1.5x the inter-quartile range).

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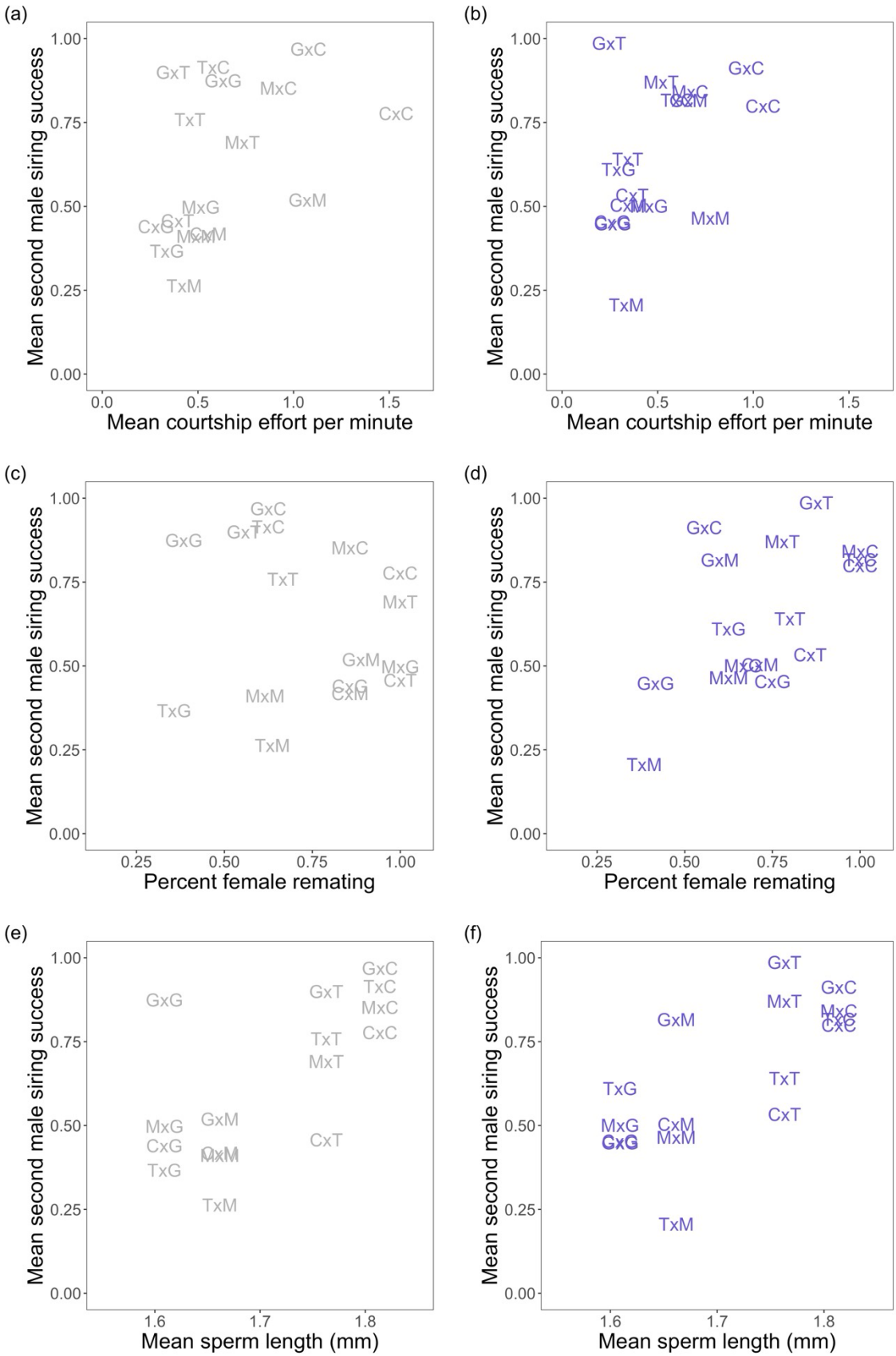
(a)
(b)
(c)
(d)
(e)
(f)
Mean second male siring success
Mean courtship effort per minute
Percent female remating
Mean sperm length (mm)
GxC
GxT
TxC
GxG
MxC
CxC
TxT
MxT
GxM
MxG
CxG
CxT
MxM
TxG
TxM

Figure 7. Second male courtship effort is significantly correlated with siring success when males are perfumed by conspecifics (a),but is not significant when perfumed by heterospecifics (b). Female remating rate is not correlated with second male siring success when males are conspecific-perfumed (c) but is correlated when males are heterospecific-perfumed (d). Sperm length is significantly correlated with siring success for both conspecific-perfumed (e) and heterospecific-perfumed males (f). Crosses are labeled as female x male genotype (G, Greece; T, Taiwan; C, California; and M, Malaysia).