

1 **Flexible polyandry in female flies is an adaptive response to infertile males**

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11 **Abbreviated title**

12 Male infertility increases polyandry

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21 **Data Accessibility Statement**

22 Analyses reported in this article can be reproduced using the data provided by Sutter *et*
23 *al.*, (2019a).

24 **Abstract**

25 Infertility is common in nature despite its obvious cost to individual fitness. Rising
26 global temperatures are predicted to decrease fertility, and male sterility is frequently
27 used in attempts to regulate pest or disease vector populations. When males are infertile,
28 females may mate with multiple males to ensure fertilisation, and changes in female
29 mating behaviour in turn could intensify selection on male fertility. Fertility assurance is
30 a potentially wide-spread explanation for polyandry, but whether and how it actually
31 contributes to the evolution of polyandry is not clear. Moreover, whether a drop in male
32 fertility would lead to a genetic increase in polyandry depends on whether females
33 respond genetically or through behavioural plasticity to male infertility. Here, we
34 experimentally manipulate male fertility through heat-exposure in *Drosophila*
35 *pseudoobscura*, and test female discrimination against infertile males before and after
36 mating. Using isogenic lines, we compare the roles of behaviourally plastic versus
37 genetically fixed polyandry. We find that heat-exposed males are less active and
38 attractive, and that females are more likely to remate after mating with these males.
39 Remating rate increases with reduced reproductive output, indicating that females use
40 current sperm storage threshold to make dynamic remating decisions. After remating
41 with fertile males, females restore normal fecundity levels. Our results suggest that male
42 infertility could explain the evolution of adaptively flexible polyandry, but is less likely
43 to cause an increase in genetic polyandry.

44 **Keywords:** sexual selection, male sterility, multiple mating, phenotypic plasticity,
45 temperature, sterile insect technique

46 **Introduction**

47 Mating failure, defined as adult females remaining unmated (Rhainds, 2010) or as the
48 failure to convert matings into reproductive success (Greenway *et al.*, 2015), is
49 pervasive in nature (Garcia-Gonzalez, 2004; Rhainds, 2010). In insects, as many as two-
50 thirds of all matings do not result in any offspring production, and the median for
51 mating failure across 30 species is 22% (Garcia-Gonzalez, 2004). Fertilisation failure
52 can also be considerable in birds (Adkins-Regan, 2015; Schmoll *et al.*, 2016) and
53 reptiles (Olsson & Shine, 1997), though estimates from wild populations remain rare.
54 Male infertility may often be responsible for mating failure. Male fertility is often
55 impaired at high temperatures (David *et al.*, 2005; Setchell, 2006; Hurley *et al.*, 2018;
56 Sales *et al.*, 2018; but see Janowitz & Fischer, 2011), and increased occurrence of heat
57 waves due to climate change (Meehl, 2004) may cause higher sterility rates (Reinhardt
58 *et al.*, 2015; Walsh *et al.*, 2019). Further, selfish genetic elements such as meiotic
59 drivers favourably target male gametes (Taylor & Ingvarsson, 2003; Price & Wedell,
60 2008), and mito-nuclear incompatibilities can devastate sperm function (Dowling *et al.*,
61 2015), meaning intra-genomic conflict is another potentially common source for a
62 reduction in male fertility. Finally, mass-sterilisation of males is a common strategy for
63 human pest control (Knipling, 1955; Dyck *et al.*, 2005).

64 Given the wide variety of factors that can create complete or partial infertility in males,
65 how should females respond? Females show adaptations that help minimise failure to
66 copulate and become inseminated (Rhainds, 2010). But copulating and/or receiving an

67 ejaculate alone will not guarantee a female successful reproduction if some males are
68 infertile. In contrast, actively choosing fertile over infertile males could allow females to
69 secure some reproductive output. The phenotype-linked fertility hypothesis posits that
70 male signals and fertility are positively correlated, allowing females to simply choose
71 attractive males to avoid reduced fertility (Sheldon, 1994). While some studies have
72 found positive correlations between male attractiveness indicators and semen quality
73 parameters (Malo *et al.*, 2005; Forstmeier *et al.*, 2017), a recent meta-analysis found no
74 general support for a link between male secondary sexual signals and tentative indices
75 of ejaculate quality (Mautz *et al.*, 2013). Even when intrinsic male fertility correlates
76 with male attractiveness, more attractive males may become sperm depleted because of
77 their increased mating success, making intrinsically more fertile males temporarily less
78 fertile (Preston *et al.*, 2001), and thus undermining the fertility benefit of female choice
79 for attractive males. The paucity of evidence for an association between male external
80 phenotype and fertility may explain why discrimination against sub-fertile or infertile
81 males is rare. For example, despite mating failure being attributable to individual seed
82 bug males (Greenway & Shuker, 2015), females do not choose fertile males (Greenway
83 *et al.*, 2017).

84 When females do not discriminate between fertile and sterile males before mating,
85 females may safeguard against mating failure simply by mating with multiple males,
86 thus making multiple mating (polyandry) an alternative to precopulatory choice (e.g.
87 Sakaluk & Cade, 1980; Gibson & Jewell, 1982; Sheldon, 1994; Arnqvist & Nilsson,
88 2000; Mossinson & Yuval, 2003; Forbes, 2014). Importantly, polyandrous females can
89 benefit even without being able to detect fertile males, as long as infertile males' sperm
90 are outcompeted by fertile males' sperm, or females remate more after mating with

91 sterile males (Lorch & Chao, 2003; Barclay, 2005; Champion de Crespigny *et al.*, 2008;
92 Hasson & Stone, 2009). Hence, increased fertility assurance for females might be a
93 major reason why polyandry is so common. Across animal taxa, 89% of all natural
94 populations investigated showed evidence for multiple paternity (Taylor *et al.*, 2014).
95 The theory underlying the evolution of polyandry for fertility assurance is well
96 developed (Hasson & Stone, 2009), and correlative studies support the notion that
97 females remate more after receiving small or infertile ejaculates (Wetton & Parkin,
98 1991; Delisle & Hardy, 1997; Torres-Vila *et al.*, 1997; Krokene *et al.*, 1998; Uller &
99 Olsson, 2005). Support through experimentally impaired male fertility, often in the
100 context of the sterile insect technique (SIT), comes from many (Miyatake *et al.*, 1999;
101 Kraaijeveld & Chapman, 2004; Gavriel *et al.*, 2009; Friesen *et al.*, 2014; Landeta-
102 Escamilla *et al.*, 2016) but not all studies (Harmer *et al.*, 2006; Abraham *et al.*, 2013;
103 Haq *et al.*, 2013; Krüger *et al.*, 2019).

104 One common limitation is that researchers have typically measured the mean response
105 of target females (Calkins & Parker, 2005 and references above). While this assesses the
106 present potential for population control through the release of sterile males, it largely
107 ignores the possibility of a dynamic female response that evolves over multiple
108 generations. Indeed, field studies of releases of sterile males into natural populations
109 have observed the evolution of precopulatory behavioural discrimination against sterile
110 males (Hibino & Iwahashi, 1991; Mcinnis *et al.*, 1996), which demonstrates the
111 importance of considering genetic variation in female mating behaviours when aiming
112 to predict evolutionary responses. Similarly, male infertility could lead to an
113 evolutionary increase in polyandry. Selection could favour either genes controlling a
114 behaviourally plastic increase in female remating after mating with infertile males, or

115 genes underlying generally polyandrous behaviour without behavioural plasticity. If
116 male sterility in natural populations is consistently high, these two scenarios have the
117 same outcome. However, if male fertility is compromised only over a temporally
118 limited period (for example, after a heatwave), a genetic response would lead to a
119 persisting increase in polyandry in the population, whereas behavioural plasticity would
120 only increase polyandry during the period of increased male sterility. To our knowledge
121 only one empirical study has explicitly addressed the evolution of female remating
122 behaviour in response to sterilised males, and did not find evidence neither for increased
123 behavioural plasticity nor increased genetically fixed polyandry after 12 generations of
124 experimental evolution in Tephritid fruit flies (Kuriwada *et al.*, 2014). However, the
125 authors concluded that insufficient genetic variation in the starting population may have
126 limited the potential for an evolutionary response (Kuriwada *et al.*, 2014).

127 Here, we investigated whether females of the fly *Drosophila pseudoobscura* mate
128 multiply to ensure successful fertilisation. Experimentally manipulating the fertility of a
129 female's first mate through heat-exposure, we measured female reproductive output in
130 the first four days following the mating, and assessed whether females are more likely
131 to remate after an infertile/sub-fertile mating. We also assessed male attractiveness and
132 courtship vigour in an attempt to infer what cues from first mates females may use to
133 make remating decisions. Importantly, using isolines that genetically differ in polyandry
134 enabled us to examine the relative roles of behavioural plasticity and genetic
135 predisposition in shaping the remating response, and hence the evolutionary potential
136 for polyandry to evolve in response to male infertility.

137 **Material and Methods**

138 Fly stocks

139 We used *D. pseudoobscura* that were collected from two populations in the Western
140 USA (Lewistown, Montana, 47°03'N, 109°28'W; Show Low, Arizona, 34°16'N,
141 110°00'W) in 2008 and 2012. We maintained all flies under a 14:10 light: dark cycle at
142 23°C, with standard *Drosophila* food vials (75 mm in height by 25 mm in width)
143 containing commercial Jazz-Mix™ *Drosophila* food (Fisher Scientific) for feeding and
144 oviposition. The experiments described here were performed between March and May
145 2018 across two experimental blocks that were shifted by three days.

146 To explicitly address the roles of behavioural plasticity and genetic variation in female
147 remating behaviour, we sourced females from ten isofemale isogenic lines that differ in
148 polyandry and that had been established using wild-caught females as described in
149 detail elsewhere (Taylor *et al*>, 2016; Sutter>*et al*>, 2019b). Briefly, offspring of wild-
150 caught females were full-sib inbred for 15 or more generations, after which flies within
151 an isoline are virtually genetically identical, and after which these isolines were
152 maintained under less-restrictive breeding conditions. Before the experiment, isolines
153 were subjected to one generation of common garden breeding. We set up five vials per
154 isoline with five virgin females and five males each, which gave females opportunity for
155 mate choice. After 24 hours, before *D. pseudoobscura* females remate (Snook & So,
156 2000), males were removed and females were transferred to a new vial to oviposit.
157 Female groups were then transferred to new food every 24 hours for 7 consecutive days.
158 We used the daughters of these females in experimental mating assays.

159 Males were derived from the same populations as the isofemale lines, but were
160 maintained across several standard *Drosophila* vials as small outbred laboratory

161 populations with overlapping generations and fluctuating population sizes. Flies
162 collected in 2008 were kept separately from flies collected in 2012, such that we
163 maintained four laboratory populations, two from both localities. Before the start of the
164 experiment, we mass-bred these small populations into large 3.5L population cages.
165 Focal males were collected from standard vials that had been left for oviposition in the
166 population cages for up to 24h.

167 Male heat-exposure treatment

168 To reduce male fertility, we exposed males to an increased temperature for a few days.
169 Heat-exposure was achieved by submerging standard vials with groups of ten males into
170 a water bath that was maintained at either the elevated temperature of 31°C or at the
171 control temperature of 23°C. About 90% of the vial volume was submerged under
172 water, such that gas exchange through a foam plug at the top of the vial was still
173 possible, but the bottom of the foam plug forced all flies to remain below the level of
174 the water surface. For logistic reasons, water baths were kept on a lab bench and thus
175 exposed to a natural diurnal light cycle. All males used had been collected within 18h of
176 eclosion and separated into single sex groups of up to 20 males. To obtain the large
177 number of virgin males needed for our mating assays we had to pool males collected
178 over several days. Thus, we heat-exposed two separate cohorts of males for each
179 experimental block. The first cohort of males (cohort A) had been kept in standard
180 conditions for 1–2 days, before they were exposed to 31°C for 72h, and finally
181 separated into individual vials and left at 23°C on the evening before the day of their
182 mating trial (i.e. around 15h before the mating trial). Because they were collected only
183 three days prior to the mating assays, the second cohort of males (cohort B) was
184 subjected to heat-exposure immediately after collection on the day of eclosion for about

185 62h until two hours before their mating trial. Thus, male cohort A was older (5–6d
186 versus 3d), exposed to heat for longer (72h versus 62h), and given more time to recover
187 from heat exposure (15h versus 2h) than cohort B. To obtain a measure of how
188 physiologically stressful our heat-exposure was to males, we measured male survival
189 during heat-exposure. To do this we counted the number of alive and dead males when
190 separating them into their individual vials at the end of their heat-exposure treatment.
191 Further, we checked whether mortality during heat-exposure led to a bias in male size,
192 i.e. favouring smaller or larger males in the heat-exposure versus the control treatment,
193 because a male size bias could in turn have affected female (re)mating patterns. As a
194 proxy for male size we measured the length of the third longitudinal vein (Taylor *et al.*,
195 2008) of one wing using *Fiji* (Schindelin *et al.*, 2012).

196 Mating assays

197 To avoid fertilisation failure, females might discriminate against sterile males before or
198 after mating by refusing to mate with sterile males or by increasing remating after
199 having mated with sterile males, respectively. Alternatively, males may provide females
200 with cues about their fertility during mating, and females may use these to make future
201 remating decisions. We used a mating assay routinely performed in our laboratory
202 (Price *et al.*, 2011; Herrera *et al.*, 2014; Taylor *et al.*, 2016) to address whether heat-
203 exposed males were less likely or slower to mate, indicating reduced male vigour or
204 attractiveness. We also determined whether heat-exposed males copulated for a shorter
205 duration, possibly indicating reduced ejaculate transfer (Price *et al.* 2008), and whether
206 these behaviours predicted female remating behaviour, potentially informing about
207 proximate mechanisms underlying polyandry. We used females from each of ten

208 isolines and males from the two populations, the temperature treatments and male
209 cohorts in a fully-factorial design.

210 We aspirated sexually mature, virgin females that were five or six days old individually
211 into vials into which a single male had been aspirated the previous day or earlier that
212 morning, depending on its cohort (see above). We took note of the time when the
213 female was introduced, and two observers scan-sampled for initiation and termination
214 of mating to record copulation latency, the duration from female introduction to the first
215 observed stable mount (i.e. the pair being relatively immobile), indicating successful
216 copulation, and copulation duration, the time from that first stable mount until the pair
217 separated. Scan-sampling meant that flies were not continuously observed, but checked
218 for copulation in short (~2min) intervals. In the second experimental block we
219 additionally recorded *ad libitum* observations of the onset of male courtship to obtain
220 data on latency to initiate courtship and time between courtship initiation and mating.
221 Observers were always blind with regards to male heat-exposure treatment and female
222 isoline identity. We used a combination of randomisation and stratification to determine
223 order in the assay to avoid time-of-day effects on mating parameters. After giving pairs
224 a minimum of two hours to mate, we removed males and froze them for later size
225 measurements. We left females that had mated to oviposit for four days, and discarded
226 females that had not mated.

227 We gave females a single opportunity to remate four days after their first mating. Again,
228 we aspirated a female into a vial containing a single 5-day old virgin male from the
229 same population as the female's first mate. These males had been kept in incubators at
230 the control temperature of 23°C. Two observers regularly scanned pairs for mating.

231 After allowing a minimum of 90min for remating, we discarded all males. To examine
232 the consequences of enforced monandry on female fitness, we denied a subset (~15%)
233 of females the opportunity to remate by aspirating the male out of the vial immediately
234 before the female was introduced. We left females to oviposit for another four days,
235 after which they were transferred to a third vial for a further four days and finally
236 discarded.

237 Fitness consequences

238 To assess the consequences of male heat-exposure and female remating for female
239 fitness, we quantified female reproductive output over 12 days, which has been shown
240 previously to correlate with lifetime reproductive success under control conditions
241 (Avent *et al.*, 2008). We counted the number of eclosed offspring from these vials 23
242 days after the first day of oviposition.

243 To obtain additional data on male fertility and mating capacity, we left a single male in
244 a vial with five virgin females for 24h, after which females were isolated and left to
245 oviposit for four days, following offspring counts after 23 days. For this small
246 experiment, we only used males from one of the populations (Show Low) from cohort B
247 in the first and from cohort A in the second experimental block, and used a haphazard
248 selection of virgin females from the ten isolines.

249 Statistical analyses

250 To test the physiological impact of heat-exposure on males and its consequences for
251 females we analysed the impact of heat-exposure on multiple aspects of male and
252 female reproductive behaviour and fitness: i) male heat-exposure survival, ii) mating

253 success, copulation latency and duration, as well as iii) female reproductive output and
254 iv) polyandry. We used R version 3.5.1 (R Core Team, 2018) for all statistical analyses
255 and figures, running binomial generalised linear mixed effects models (GLMM) and
256 linear mixed effects models (LMM) implemented in *lme4* version 1.1-14 (Bates *et al.*,
257 2015), and zero-inflated mixed models in *glmmTMB* (Brooks *et al.*, 2017). Descriptive
258 statistics and sample sizes for the different response variables are summarised in
259 Table 1. Here we give an overview of the fixed and random predictor variables included
260 in the different models (see also Tables 2, 3 & S1–S4).

261 i) We first measured male survival to assess how physiologically stressful our heat-
262 exposure treatment was: We ran a binomial GLMM with heat-exposure, male
263 cohort, their interaction and block as fixed effects, and post-eclosion housing vial
264 and population as random intercepts. To ask whether survival was biased with
265 respect to male size, we ran an LMM on the wing size of surviving males, with
266 heat-exposure, male cohort and their interaction as fixed effects, and male
267 collection batch (16 unique block, population, and collection day combinations) as
268 a random effect.

269 ii) We measured male mating success, copulation latency and duration to test for
270 effects of heat-exposure on male reproductive performance: We ran a binomial
271 GLMM for mating success as well as LMMs on log-transformed copulation
272 latency and duration with heat-exposure, male cohort, their interaction, block,
273 female age, male size and temporal order within the mating assay as fixed effects,
274 and random intercepts for female post-eclosion housing vial, female isoline and
275 male population.

276 iii) We then tested the consequences of mating with a heat-exposed male with or
277 without successive remating with control males for female reproductive output:
278 Because many of the oviposition vials contained no offspring, we used zero-
279 inflated models with a Gaussian distribution for the conditional part implemented
280 in *glmmTMB* (Brooks *et al.*, 2017), and examined residuals with *DHARMA*
281 (Hartig, 2018). Our conditional full model included heat-exposure, female
282 remating, male size, laying vial and two- and three-way interactions as fixed
283 effects. We included random intercepts for female ID, female isoline, male
284 collection batch (see above), and random slopes for individual females to account
285 for repeated measures across a female's three laying vials. (Schielzeth &
286 Forstmeier, 2009). Our zero-inflated full model included heat-exposure, female
287 remating, male cohort, laying vial and two- and three-way interactions as fixed
288 effects.

289 iv) Finally, we asked what explained variation in polyandry: We ran a binomial
290 GLMM with fixed effects for heat-exposure, reproductive output from the first
291 oviposition vial and male size including two-way interactions with heat-exposure,
292 and female age and temporal order within the mating assay. We included random
293 intercepts for female isoline and male collection batch as random intercepts.
294 Because of our explicit interest in distinguishing between behavioural plasticity
295 and genetic polyandry, we additionally included the interaction between first male
296 temperature treatment and female isoline as an additional random effect (i.e.
297 random slopes for isolines).

298 Whenever possible, we extracted effect sizes and p values from full models to avoid
299 biasing effect sizes through the removal of non-significant terms (Forstmeier &

300 Schielzeth, 2011). P values for fixed effects from LMMs were obtained from *t*-tests
301 using the Kenward-Roger approximation for denominator degrees of freedom
302 implemented in *lmerTest* (Kuznetsova *et al.*, 2016). For reproductive output, we ran a
303 large albeit not exhaustive selection of combinations of full and reduced conditional and
304 zero-inflation models, and selected the best model based on the lowest AIC value. To
305 facilitate the interpretation of main effects in the presence of interactions and to aid
306 model convergence, we centred covariates to a mean of zero. Age covariates were
307 mean-centred, and temporal order within an assay (pairs that were set-up earlier had
308 more time available for mating/remating) was centred and scaled to a standard deviation
309 of one. For models on mating behaviour, we additionally centred contrasts between two
310 factors (male cohorts A and B, first and second experimental blocks) by coding factor
311 levels as minus 0.5 and 0.5, respectively (Schielzeth, 2010). Approximate 95%
312 confidence intervals (*CI*) for effect sizes were taken as twice the standard error either
313 side of the mean (Crawley, 2007). We tested significance of random effects using
314 likelihood ratio tests between models including and excluding the variable of interest
315 (Bolker *et al.*, 2009). Additionally, we estimated among-isoline variances and the
316 covariance between polyandry after mating with control and heat-exposed males using a
317 Bayesian approach implemented in *MCMCglmm* (Hadfield, 2010; see the
318 supplementary material).

319 **Results**

320 Heat-exposure reduces male survival and mating success

321 Heat exposure decreased male survival substantially in male cohort A, but only
322 marginally in cohort B (Table 1; Fig S3). Survival was lower than 50% in cohort A

323 heat-exposed males but higher than 97% in the three other treatment-cohort
324 combinations, manifested as a highly significant interaction between treatment and male
325 cohort (GLMM, N = 1515, effect size β [95%CI] on logit scale = -3.8 [-5.9;-1.7], $z = -$
326 3.58, $p < 0.001$; Table S2). There was no indication that heat-exposure caused size-
327 dependent mortality, as the interaction between temperature and male cohort did not
328 significantly explain variation in body size of surviving males (i.e. wing length; LMM,
329 N = 925, $\beta = -0.01$ [-0.03;0.02], $t_{1,907.6} = -0.58$, $p = 0.565$; Table S3). Substantial
330 variation in body size was explained by pre-eclosion conditions (unique combinations
331 of populations, male cohorts and experimental blocks; likelihood ratio test LRT,
332 $\chi^2(14) = 2.6$, $p < 0.001$) but not by post-eclosion treatment (heat-exposure; $p > 0.5$).

333 Males that had been heat-exposed were much less likely to mate (binomial GLMM,
334 N = 916, $\beta = -3.1$ [-3.6;-2.7], $z = -14.2$, $p < 0.001$; Table S2). Mating success was 86%
335 in control males but only 30% in heat-exposed males (Fig 1, Table 1). In conjunction
336 with a decrease in mating success, copulation latency of successful males was longer for
337 heat-exposed males (log-transformed latency in minutes; LMM, N = 496, $\beta = 1.1$
338 [0.8;1.3], $t_{1,459.2} = 9.4$, $p < 0.001$; Fig 1 & S1; Table S1). Copulations with heat-exposed
339 males were shorter than those with control males (LMM, N = 487, $\beta = -0.25$ [-0.35;-
340 0.15], $t_{1,451.1} = 9.4$, $p < 0.001$; Table 1 & S1; Fig S1). Additional data on male courtship
341 collected only in the second experimental block indicated that heat-exposed males were
342 slower and less likely to initiate courtship, and that their courtship quality or intensity
343 may have been inferior to that of control males (see supplementary Results, Table S1 &
344 Fig S1).

345 Male heat-exposure reduces female reproductive fitness

346 Females mated to heat-exposed males had lower reproductive fitness than females
347 mated to control males. This was true both for the likelihood of failing to produce any
348 offspring over four days after mating as well as for the number of offspring produced
349 among the subset of females that did produce offspring (Fig 2). In our main dataset, this
350 was evidenced by a significant baseline effect of male heat exposure treatment on the
351 zero-inflation model ($N = 498$, $\beta = 6.5$, $z = 8.4$, $p < 0.001$) as well as the conditional
352 model ($\beta = -28.9$ [-44.6 ; -13.2], $z = -3.9$, $p < 0.001$; Table 2). In our additional, small
353 dataset, where we housed males with five females for 24h, heat-exposed males
354 successfully reproduced with fewer females (binomial GLM, $\beta = -2.9$, $z = -6.8$,
355 $p < 0.001$), and sired marginally fewer offspring per fertile mating (LM, $\beta = -13.0$ [$-$
356 25.5 ; 0.5], $t_{1,29} = 4.3$, $p = 0.046$; Table 1).

357 Polyandry restores female reproductive fitness in the face of male infertility

358 Polyandry had a beneficial effect on reproductive fitness of females previously mated to
359 heat-exposed males (Table 3), mainly through reducing the incidence of complete
360 reproductive failure (Fig 2 & Table 2). In contrast, polyandry had no substantial effect
361 on fecundity under control conditions (Fig 2), consistent with a recent study (Sutter *et*
362 *al*>, 2019b). Females with higher initial reproductive output were less likely to remate
363 (chosen monandry; see below), but appeared to run out of sperm over the next 4–8 days
364 (Fig 2). The temporal decline in reproductive fitness of facultatively monandrous
365 females and the reproductive increase in polyandrous females within the male heat-
366 exposure treatment contrasted with the consistent temporal patterns within the control
367 treatment. This explained the three-way interaction between heat-exposure treatment,
368 remating phenotype and oviposition vial.

369 Phenotypically plastic polyandry

370 Four days after their first mating, females that had mated with a heat-exposed male were
371 twice as likely to remate (84%) as were females that had mated with control males
372 (42%; Table 1). The relationship between polyandry and reproductive output after the
373 first mating suggests the difference in mating behaviour is causally related to reduced
374 fertility and fecundity. Females were more likely to remate if they had produced fewer
375 offspring after the first mating (binomial GLMM, $N = 427$, $\beta = -0.4$ $[-0.7; -0.1]$, $z = -$
376 2.5 , $p = 0.012$; Table 3). However, when matched for fecundity, females mated to heat-
377 exposed males still had a higher remating likelihood ($\beta = 1.9$ $[1.1; 2.7]$, $z = 4.7$,
378 $p < 0.001$; Table 3). Polyandry tended to decrease after mating with larger males and to
379 increase with female age (Table 3).

380 The increase in polyandry after mating with heat-exposed males was consistent in
381 females from all ten isolines, indicated by the interaction between female isoline and
382 heat-exposure of the first mate not explaining a significant amount of variation in
383 polyandry (Fig 3; LRT, $\chi^2(2) = 0.85$ $p = 0.654$). In contrast, significant variation
384 between isolines confirmed genetic variation in polyandry (LRT, $\chi^2(1) = 10.4$
385 $p = 0.001$). However, our additional analyses using MCMCglmm (Hadfield, 2010)
386 showed this genetic variation was substantial in control females but negligible in
387 females mated to heat-exposed males (supplementary Results). Moreover, there was no
388 clear correlation between polyandry of isolines after mating with control versus heat-
389 exposed males. In combination, this meant we were unable to confidently reject that
390 there is genetic variation in behavioural plasticity, nor could we confidently conclude
391 that the response of isolines was quantitatively consistent. Our results indicate

392 behavioural plasticity in polyandry, and genetic variation in polyandry, but show no
393 clear evidence for genetic variation in behavioural plasticity.

394 **Discussion**

395 Here we show that females representing distinct genotypes consistently use polyandry
396 as a behaviourally flexible strategy to mitigate the potential fitness loss arising from
397 male sterility, using cues from stored ejaculates. We found no clear evidence for genetic
398 variation in how females respond to male infertility, but the flexible female response we
399 describe here could intensify selection on male fertility, and aid population resilience.

400 Adaptively flexible polyandry

401 After mating with heat-exposed males with severely compromised fertility, female
402 remating doubled from 42% to 84%. Safeguarding against male infertility is a potential
403 adaptive explanation for the ubiquity of female multiple mating, and a number of
404 studies have reported increased polyandry after mating with experimentally sterilised
405 males (e.g. medfly: Miyatake *et al.*, 1999; Kraaijeveld & Chapman, 2004; Gavriel *et al.*,
406 2009; red garter snake: Friesen *et al.*, 2014; *Anastrepha serpentina*: Landeta-Escamilla
407 *et al.*, 2016), further supported by correlational data (Sakaluk & Cade, 1980; Wetton &
408 Parkin, 1991; Uller & Olsson, 2005; Reding, 2015; but see Morrow *et al.*, 2002). Other
409 experiments however found no effect of male sterility on female remating behaviour
410 (Queensland fruit fly: Harmer *et al.*, 2006; *Anastrepha fraterculus*: Abraham *et al.*,
411 2013; melon fly: Haq *et al.*, 2013; *Drosophila suzukii*: Krüger *et al.*, 2018). A potential
412 explanation for this discrepancy is that the latter studies used artificial techniques such
413 as genetic manipulation and irradiation to induce male sterility, and these males may

414 lack the cues present in naturally sterile males, with which female remating behaviour
415 has coevolved.

416 Heat-induced male infertility is likely to be relevant in nature (Sales *et al.*, 2018; Walsh
417 *et al.*, 2019), and should create a strong incentive for female multiple mating. Here,
418 more than half of the females that mated with heat-exposed males produced no
419 offspring following mating, indicating high rates of male sterility, compared to a mere
420 five percent in the control group. Among these females with failed early reproduction,
421 remating rates were as high as the proportion of virgin females that mated with control
422 males, meaning the effect of heat-exposure on polyandry could have been driven by
423 pseudopolyandry rather than true polyandry (Fisher *et al.*, 2013). However, when
424 focusing on the subsets of females that had non-zero early reproductive output, the
425 difference in polyandry between females mated to heat-exposed versus control males
426 was again almost two-fold (76% [N = 66] versus 39% [N = 274]). More formally, in our
427 analysis on polyandry where we included early reproductive output as a predictor
428 variable, male heat-exposure showed a very strong effect on polyandry (Table 3, see
429 also Fig S2).

430 Females may have used information obtained during the first mating to make remating
431 decisions. Heat-exposure decreased survival only in male cohort A, but had pronounced
432 sub-lethal effects on sexual behaviour that were similar in both male cohorts. Heat-
433 exposed males were slower to initiate courtship, took longer to be accepted by females
434 and copulated for a shorter duration, possibly because heat-exposure had negative
435 effects on male condition, thus providing females with additional pre- and peri-
436 copulatory cues about male fertility. However, remating likelihood was not related to

437 copulation latency or duration of a female's first mating (Table S6), making it more
438 likely that females used cues from stored ejaculates. Our experimental design did not
439 distinguish between whether changes in sperm or seminal fluids were responsible for
440 the increase in polyandry. Either mechanism is plausible, but the effects are likely to be
441 species-specific. For example, sperm-less males can induce a refractory period in
442 female Queensland fruit flies and Medflies (Harmer *et al.*, 2006; Gabrieli *et al.*, 2016),
443 but both seminal fluids and sperm are required for inhibiting remating in *Anastrepha*
444 *fraterculus* and *A. ludens* (Abraham *et al.*, 2016), and *Drosophila melanogaster* flies
445 (Liu & Kubli, 2003). Independent of the precise mechanism, our results suggest that
446 polyandry is not simply a response to the absence of fertile sperm but that females take
447 current semen storage into account when making remating decisions (Manning, 1967;
448 Crudgington *et al.*, 2005).

449 Behavioural plasticity appeared to be more important than genetic variation in
450 polyandry. Polyandry increases with latitude across *D. pseudoobscura* populations in
451 North America, consistent with the proximate effect of lower temperature increasing
452 polyandry (Taylor *et al.*, 2016). But variation in polyandry between populations is
453 genetic and not simply explained by these proximate effects (Taylor *et al.*, 2016).
454 Similarly, the genetic cline is opposite to that expected if polyandry had evolved in
455 response to higher rates of heat-induced male sterility. More generally, variation in male
456 fertility could have favoured the evolution of behavioural plasticity in polyandry. Using
457 females from distinct genetic backgrounds that differ in polyandry (Taylor *et al.*, 2016;
458 Sutter *et al.*, 2019b), we found that females from all backgrounds substantially
459 elevated polyandry levels after mating with sub-fertile males, suggesting behavioural
460 plasticity was largely independent of genetic variation in polyandry. Including

461 reproductive output as a covariate meant our tests were controlled for variation in
462 reproductive output among isolines (see above). Unfortunately, our power to detect a
463 potential subtle genotype-by-treatment interaction for polyandry was limited by the low
464 mating success of heat-exposed males (Fig 3 & Table S5). This means we cannot
465 comprehensively rule out that there may be genetic variation in behavioural plasticity of
466 polyandry. Selection may in general favour females that make reproductive decisions
467 dynamically and flexibly (Gowaty, 2013; Ah-King & Gowaty, 2016). In the context of
468 male infertility, females appear to update their remating decisions according to their
469 current state (Gowaty & Hubbell, 2009), and to indeed dynamically lower their mate
470 acceptance threshold when sperm storage is low.

471 Consequences for populations

472 Plastically elevated polyandry levels have important implications for population
473 viability (Holman & Kokko, 2013), particularly for populations under threat due to
474 rising male infertility, and for targets of the sterile insect technique (SIT). First, climate
475 change means that many organisms are likely to face increased male fertility problems
476 (Walsh et al., 2019). If females increase remating after mating with infertile males, heat-
477 induced male infertility may have little impact on population productivity as long as
478 there are enough fertile males. Little is known about the heritability of temperature
479 sensitivity of male fertility (Walsh et al., 2019). But, if variation in male fertility is
480 heritable and continuous, more intense postcopulatory sexual selection due to increased
481 polyandry (Morimoto *et al.*, 2019) will increase reproductive skew towards fully fertile
482 males, which may accelerate adaptation to increasing temperatures and delay population
483 extinction (Parrett & Knell, 2018). Second, plastically elevated polyandry thwarts
484 population control attempts through SIT (Kraaijeveld & Chapman, 2004; Barclay,

485 2005). Thus, understanding short-term plasticity in polyandry as well as the amount of
486 genetic variation underlying this plasticity is important for predicting the potential of
487 SIT. For example, even if the average female shows no increased remating after mating
488 with sterile males, populations may still harbour genetic variation in female remating
489 behaviour. This would lead to an increase in polyandry in response to SIT across
490 generations, hence hampering SIT effectiveness.

491 Conclusions

492 Mating failure is common, and represents a potential explanation for the ubiquity of
493 female multiple mating. Male fertility is often compromised by natural processes and
494 human intervention. Here, we have shown that females flexibly adjusted their remating
495 rate according to their demands for fertile sperm, consistent with behavioural plasticity
496 that was largely independent of genetic variation in polyandry. Polyandry allowed
497 females to buffer against fitness costs associated with mating with heat-exposed males
498 with low fertility, which may hamper the impact of release of sterile males for
499 population control, but may increase selection on male fertility and assist adaptation to
500 increasing global temperatures.

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726

727 **Figure legends**

728 **Figure 1:** Male mating success and latency. Heat-exposed males (red) had a longer
729 copulation latency and reduced mating success compared to control males (blue; see
730 main text and Table S1). Thin lines represent approximate 95% confidence intervals
731 from a cox proportional hazard model on right-censored mating latency with other fixed
732 effects centred. Note the log-scale of the x axis.

733 **Figure 2:** Male heat-exposure reduces female reproductive output, but polyandry can
734 restore fitness. Framed circles and error bars depict mean and approximate 95%
735 confidence intervals. Faint circles represent raw data, with circle area proportional to the
736 number of observations. Under enforced monandry, females mated to heat-exposed
737 males had consistently low reproductive fitness (left panel). Females often chose not to
738 remate when initial reproductive output was substantial after mating with heat-exposed
739 males, but soon after showed reduced reproductive output (central panel). Remating
740 with fertile males fully restored subsequent reproductive fitness in females that had
741 mated with heat-exposed males (right panel).

742 **Figure 3:** Females increase polyandry after mating with heat-exposed males through
743 behavioural plasticity. Isolines were assigned a colour gradient according to polyandry
744 at the control temperature. Polyandry was consistently higher after mating with heat-
745 exposed males (right) versus control males (left; Table 3). The area of circles is
746 proportional to the sample size. Raw values and sample sizes are given in Table S5.
747 Note the smaller sample sizes for females first mated to heat-exposed males due to low
748 mating success of heat-exposed males, limiting the power to detect genetic variation in
749 behavioural plasticity.

750 **Table 1:** Summary statistics and sample sizes.

Temperature	Control (23°C)		Heat-exposure (31°C)		Heat effect	Full model	Illustration
	A	B	A	B			
Male cohort							
<i>main experiment</i>							
Male mortality (N)	2% (285)	0.8% (260)	52% (460)	2% (510)	(↑)	Table S2	Fig S3
Mating success (N)	91% (163)	84% (230)	24% (148)	33% (381)	↓	Table S1	Fig 1
Copulation latency [min] (N)	3.4±3.2 (147)	8.2±17.5 (192)	16.5±25.8 (36)	18.5±23.6 (124)	↑	Table S1	Fig 1 & S1
Copulation duration [sec] (N)	6.6±2.3 (148)	5.9±1.8 (194)	4.9±2.0 (36)	5.7±5.1 (123)	↓	Table S1	Fig S1
4d fecundity (N)	42.3±19.8 (147)	43.2±19.0 (192)	33.3±25.0 (35)	9.8±18.6 (125)	↓	Table 2	Fig 2
Polyandry (N)	44% (147)	40% (136)	77% (35)	85% (109)	↑	Table 3	Fig 3
<i>additional males</i>							
Male fertility (N)	4.8±0.7 (9)	3.1±1.2 (8)	2.0±1.3 (11)	0.5±0.7 (11)	↓		
4d fecundity (N)	229±65 (9)	131±63 (8)	78±38 (9)	41±26 (5)	↓		

751 Given are mean, standard deviation and sample sizes for survival, mating behaviours and reproductive output. The effect of male heat-
 752 exposure is indicated by arrows. For detailed results see the full models as indicated in the last column.

753 **Table 2:** Model summary for female reproductive output.

<i>Fixed/Random effects</i>	Conditional model				Zero-inflation model					
	Coef	SE (Coef)	z	p	Var	SD	Coef	SE (Coef)	z	p
Intercept [control; forced monandry; Vial A (d1–5)]	42.777	2.775	15.41	<0.001			–5.624	0.942	–5.97	<0.001
Heat-exposure	–28.826	7.456	–3.87	<0.001			6.509	0.778	8.37	<0.001
Chosen monandry (Mono)	3.169	2.544	1.25	0.213			–0.648	1.020	–0.64	0.525
Chosen polyandry (Poly)	0.894	2.705	0.33	0.741			3.876	0.904	4.29	<0.001
First mate's size (centred & scaled)	–1.106	0.565	–1.96	0.050						
Vial B (d5–9)	–16.407	3.038	–5.40	<0.001			2.395	0.934	2.57	0.010
Vial C (d9–13)	–6.657	3.186	–2.09	0.037			3.512	0.918	3.83	<0.001
Male cohort (A)							–0.705	0.226	–3.12	0.002
Heat:Mono	30.004	8.566	3.50	<0.001			–0.632	0.725	–0.87	0.383
Heat:Poly	17.664	7.976	2.22	0.027			–4.237	0.696	–6.09	<0.001
Heat:Vial B	28.646	11.099	2.58	0.010			–2.425	0.636	–3.81	<0.001
Heat:Vial C	0.711	12.416	0.06	0.954			–3.140	0.558	–5.63	<0.001
Mono:Vial B	–5.725	3.514	–1.63	0.103			0.772	0.949	0.81	0.416
Mono:Vial C	–11.299	3.683	–3.07	0.002			0.897	0.977	0.92	0.358
Poly:Vial B	–4.031	3.725	–1.08	0.279			–3.312	0.854	–3.88	<0.001

Table 2 (continued)

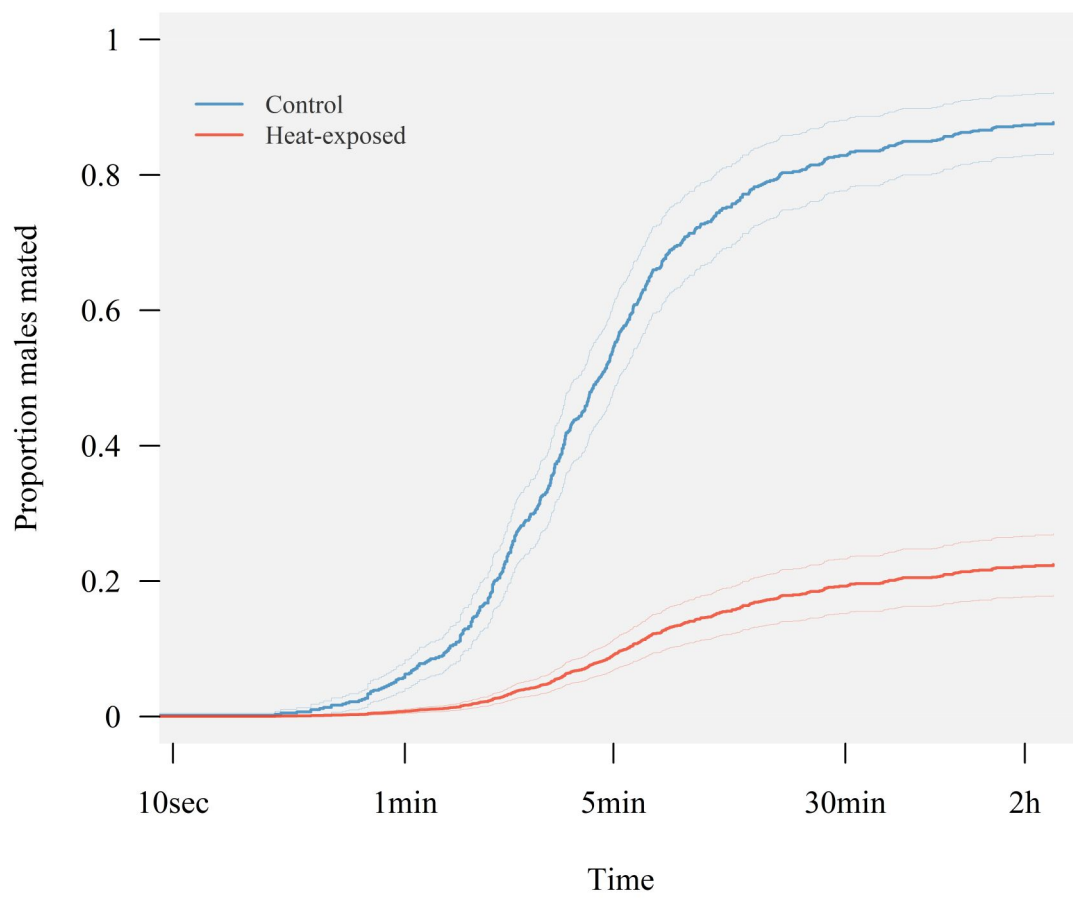
Fixed/Random effects	Conditional model				Zero-inflation model					
	Coef	SE (Coef)	z	p	Var	SD	Coef	SE (Coef)	z	p
Poly:Vial C	-6.831	3.914	-1.75	0.081			-3.287	0.850	-3.87	<0.001
Heat:Mono:Vial B	-32.379	12.860	-2.52	0.012						
Heat:Mono:Vial C	-21.605	14.467	-1.49	0.135						
Heat:Poly:Vial B	-15.155	11.634	-1.30	0.193						
Heat:Poly:Vial C	12.732	12.929	0.99	0.325						
<i>Individual female</i>					7.70	2.77				
<i>Female:Vial (random slopes)</i>					<0.01	0.02				
<i>Female isoline (10 levels)</i>					26.17	5.12				
<i>Male collection batch (16 levels)</i>					3.59	1.90				
<i>Residual</i>					245.20	15.66				

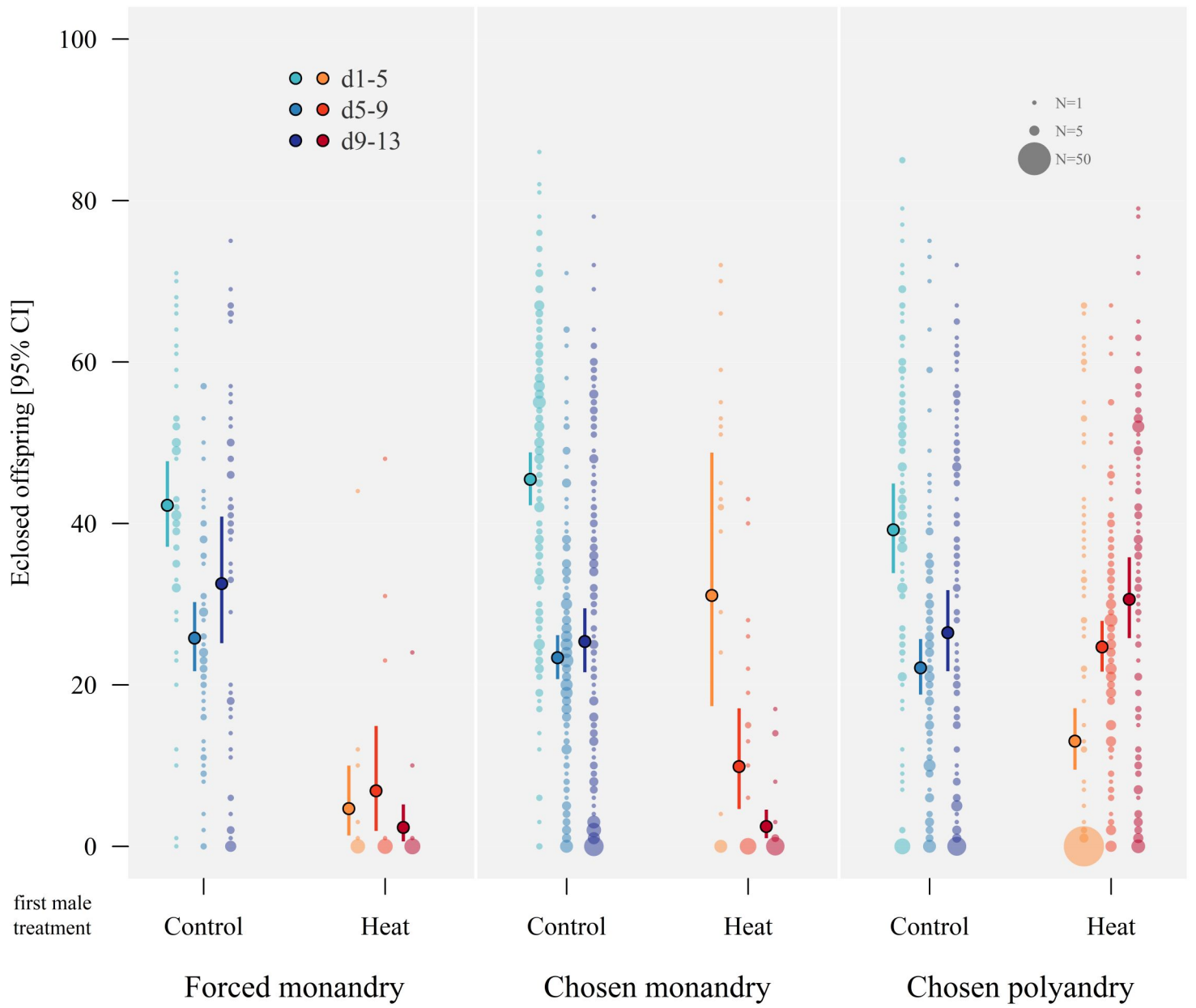
754 The conditional model describes the Gaussian component of female reproductive output (498 females) while the zero-inflation model
755 accounts for the likelihood of reproductive failure. The model with the lowest AIC value was chosen as the best model. See Table S4 for an
756 overview of models and associated AIC values.

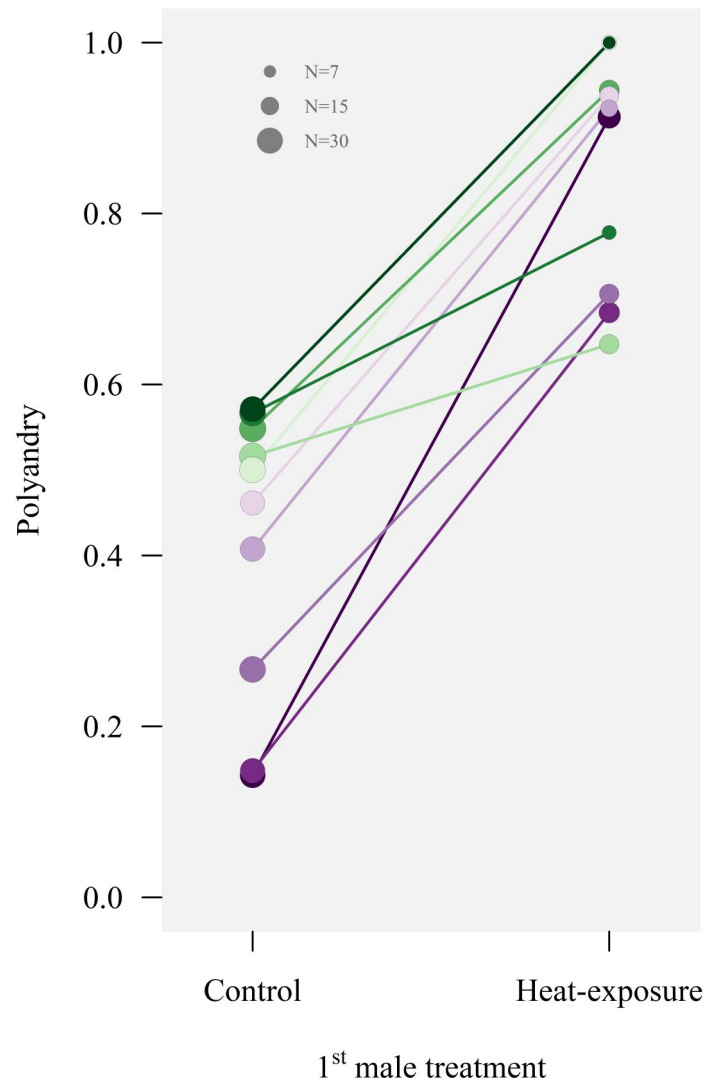
757 **Table 3:** Full model summary for polyandry.

binomial GLMM (N = 427)						
Fixed/Random effects	Coef	SE (Coef)	z	p	Var	SD
Intercept (control temperature)	-0.163	0.246	-0.66	0.508		
Heat-exposure	1.910	0.408	4.68	<0.001		
4d reproductive output (centred & scaled)	-0.411	0.164	-2.51	0.012		
First mate's size (centred & scaled)	-0.220	0.136	-1.62	0.105		
Female age (centred)	0.513	0.271	1.89	0.059		
Order in assay (centred & scaled)	0.097	0.145	0.67	0.504		
Heat:Reproductive_output	-0.337	0.295	-1.14	0.253		
Heat:First_mate_size	-0.650	0.348	-1.87	0.062		
<i>Male collection batch (16 levels)</i>					<0.001	<0.001
<i>Female isoline (10 levels)</i>					0.38	0.62
<i>Heat:Female_isoline (random slopes)</i>					0.21	0.46

758 Four-day reproductive output corresponds to the number of offspring eclosed from the
 759 vial in which a female was housed between her first mating and the remating
 760 opportunity. Random slopes for female isolines were included to test for genetic
 761 variation in behavioural plasticity (G x E; see Fig 3).







Electronic supplementary material

Adaptively flexible polyandry by female flies when males are infertile

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Supplementary results:

Male courtship behaviour

To investigate whether reduced mating success for heat-exposed males was caused by female discrimination against heat-exposed males or reduced courtship by heat-exposed males, we recorded and analysed data on courtship latency in the second experimental block. Courtship latency was longer for heat-exposed males. This was true both for eventually successful and unsuccessful males (Table 2; Fig S1). Additionally, of the males that did not mate, heat-exposed males were more likely not to have been observed courting (71% versus 50% for control males). And in the subset of males that were observed to both court and mate, latency from courtship initiation to mating tended to be longer for heat-exposed males (3.6 versus 1.5min), though the effect was not statistically significant, probably because of the small sample size (N = 52). In combination, these results suggest that heat-exposed males were slower and less likely to initiate courtship, and that their courtship quality or intensity may have been inferior to that of control males.

Estimating genetic variation in polyandry and behavioural plasticity

In addition to simply testing for significant effects of female isoline and its interaction with male treatment on polyandry (described in the main text), we used a bivariate model in MCMCglmm to estimate among-isoline variances, and covariance between polyandry of females that had mated with control or with heat-exposed males. For fixed effects (specified in Table 3) and the random effect associated with female isoline, we fitted an unstructured variance-covariance matrix that allows estimation of covariances between parts of the model. We fitted variances but no covariances for the random effect male collection batch. We fixed the residual variance for polyandry (binary outcome) at 10, and rescaled random effect variance estimates as $Var/(1 + c2 * 10)$, where $c2 = ((16 * \sqrt{3})/(15 * \pi))^2$, following Jarrod Hadfield's MCMCglmm course notes (<https://cran.r-project.org/web/packages/MCMCglmm/vignettes/CourseNotes.pdf>). The model was run for 4,050,000 iterations with a thinning interval of 2000 and a burn-in of 50,000 with parameter-inflated priors. This resulted in 2000 samples from the posterior for which autocorrelation between successive samples for parameters was less than 0.1.

Female isoline explained a substantial proportion of variation in polyandry of females after mating with control males (posterior mode [95% credible interval] = 0.12 [0.025, 0.39]), whereas very little variation in polyandry of females mated with heat-exposed males was explained by isoline identity (0.002 [<0.0001 , 0.42]). Finally, there was no clear correlation (estimated from (co)variances) between isoline female behaviour after mating with control or heat-exposed males (0.31 [-0.55, 0.88]; note the very large credible interval).

Table S1: Model summaries for mating behaviours

Fixed/Random effects	Mating success (binomial GLMM; N = 916)						Copulation latency (log LMM; N = 496)						Copulation duration (log LMM; N = 487)					
	Coef	SE (Coef)	z	p	Var	SD	Coef	SE (Coef)	t	p	Var	SD	Coef	SE (Coef)	t	p	Var	SD
Intercept (control)	2.078	0.223	9.31	<0.001	-	-	5.248	0.104	50.70	<0.001	-	-	5.866	0.060	97.71	<0.001	-	-
Heat-exposure	-3.116	0.219	-14.21	<0.001	-	-	1.056	0.113	9.37	<0.001	-	-	-0.252	0.051	-4.99	<0.001	-	-
Cohort (B→A; centred)	0.357	0.340	1.05	0.294	-	-	-0.446	0.113	-3.94	<0.001	-	-	0.109	0.051	2.14	0.033	-	-
Heat:Cohort	-0.980	0.406	-2.41	0.016	-	-	0.233	0.221	1.05	0.293	-	-	-0.168	0.099	-1.69	0.092	-	-
Block (centred)	-0.548	0.185	-2.96	0.003	-	-	0.101	0.099	1.03	0.308	-	-	0.110	0.044	2.49	0.014	-	-
Female age (centred)	-0.040	0.218	-0.18	0.855	-	-	-0.182	0.121	-1.51	0.134	-	-	-0.082	0.054	-1.51	0.134	-	-
Male size (centred & scaled)	0.324	0.097	3.36	0.001	-	-	-0.035	0.053	-0.65	0.514	-	-	-0.036	0.024	-1.51	0.132	-	-
Order in assay (centred & scaled)	-0.346	0.092	-3.75	<0.001	-	-	0.055	0.048	1.15	0.255	-	-	0.007	0.021	0.34	0.735	-	-
Mating (yes vs no)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Female housing vial (≤ 93 levels)	-	-	-	-	0.059	0.244	-	-	-	-	<0.001	<0.001	-	-	-	-	<0.001	<0.001
Female isoline (10 levels)	-	-	-	-	0.061	0.247	-	-	-	-	0.009	0.096	-	-	-	-	<0.001	<0.001
Male population (4 levels)	-	-	-	-	0.052	0.227	-	-	-	-	0.027	0.164	-	-	-	-	0.012	0.109
Residual	-	-	-	-	-	-	-	-	-	-	0.987	0.994	-	-	-	-	0.197	0.444

Fixed/Random effects	Courtship latency (log LMM; N = 127)						Courtship duration (log LMM; N = 52)					
	Coef	SE (Coef)	t	p	Var	SD	Coef	SE (Coef)	t	p	Var	SD
Intercept (control)	5.924	0.266	22.29	<0.001	-	-	4.531	0.341	13.29	<0.001	-	-
Heat-exposure	1.191	0.289	4.11	<0.001	-	-	0.764	0.562	1.36	0.180	-	-
Cohort (B→A; centred)	-0.521	0.376	-1.38	0.169	-	-	-	-	-	-	-	-
Heat:Cohort	1.079	0.521	2.07	0.041	-	-	-	-	-	-	-	-
Block (centred)	-	-	-	-	-	-	-	-	-	-	-	-
Female age (centred)	-0.107	0.315	-0.34	0.743	-	-	-	-	-	-	-	-
Male size (centred & scaled)	-0.098	0.143	-0.69	0.496	-	-	-0.219	0.290	-0.76	0.454	-	-
Order in assay (centred & scaled)	0.313	0.124	2.53	0.013	-	-	-	-	-	-	-	-
Mating (yes vs no)	-0.933	0.276	-3.38	0.001	-	-	-	-	-	-	-	-
Female housing vial (≤ 93 levels)	-	-	-	-	-	-	-	-	-	-	-	-
Female isoline (10 levels)	-	-	-	-	0.016	0.128	-	-	-	-	-	-
Male population (4 levels)	-	-	-	-	<0.001	<0.001	-	-	-	-	<0.001	<0.001
Residual	-	-	-	-	1.438	1.199	-	-	-	-	3.767	1.941

Table S2: Full model summary for male survival under heat-exposure and control temperature. Vials containing groups of up to ten males were submerged in water baths at 23°C or 31°C for two-and-a-half (cohort B; see main text) or three days (cohort A). Experimental block was centred as described in the main text. Effects associated with a p value smaller than 0.05 are highlighted in bold.

binomial GLMM (N = 1645)						
Fixed/Random effects	Coef	SE (Coef)	z	p	Var	SD
Intercept (control; cohort B)	5.570	0.813	6.85	<0.001	-	-
Heat-exposure	-1.053	0.869	-1.21	0.226	-	-
Cohort (A)	-0.833	0.941	-0.89	0.376	-	-
Heat:Cohort	-3.770	1.053	-3.58	<0.001	-	-
Block (centred)	0.929	0.349	2.67	0.008	-	-
<i>Housing vial (155 levels)</i>	-	-	-	-	1.51	1.23
<i>Population (4 levels)</i>	-	-	-	-	<0.001	<0.001

Table S3: Full model summary for male size (length of wing L3 [mm]). Only males surviving the heat-exposure/control were measured. Virgin collection batch corresponds to unique combinations of virgin collection day and population cage.

LMM (N = 925)							
Fixed/Random effects	Coef	SE (Coef)	ddf	t	p	Var	SD
Intercept (control; cohort B)	1.435	0.020	15.2	73.182	<0.001	-	-
Heat-exposure	-0.004	0.010	907.0	-0.569	0.569	-	-
Cohort (A)	0.021	0.028	15.8	0.783	0.445	-	-
Heat:Cohort	-0.008	0.013	907.6	-0.576	0.565	-	-
<i>Virgin collection batch (16 levels)</i>	-	-	-	-	-	0.003	0.053
<i>Residual</i>	-	-	-	-	-	0.008	0.091

Table S4: Overview of models for reproductive output. Models were run using glmmTMB and were sorted along ascending AIC values. All conditional models included random intercepts for female ID, female isoline, male collection batch (unique combinations of population, cohort and block), and random slopes for individual females across the three laying vials (see main text).

	Conditional model										Zero-inflation model								df	AIC	ΔAIC	
	Intercept	Heat-exposure	Mating (FM, Mono, Poly)	First mate's size	Vial (A-C)	Heat:Mating	Heat:Vial	Mating:Vial	Heat:Size	Heat:Mating:Vial	Intercept	Heat-exposure	Mating (FM, Mono, Poly)	Vial (A-C)	Male cohort	Heat:Mating	Heat:Vial	Mating:Vial				Heat:Mating:Vial
Model 1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	39	11648.5	0.0
Model 2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	40	11650.1	1.6
Model 3	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	38	11650.3	1.9
Model 4	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	42	11654.6	6.1
Model 5	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	41	11662.5	14.0
Model 6	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	34	11664.5	16.0
Model 7	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	32	11668.0	19.5
Model 8	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	38	11668.7	20.2
Model 9	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	32	11671.9	23.4
Model 10	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	36	11672.3	23.8
Model 11	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	30	11674.6	26.1
Model 12	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	36	11676.2	27.7
Model 13	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	30	11677.0	28.6
Model 14	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	28	11678.7	30.2
Model 15	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	34	11679.3	30.9
Model 16	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	34	11681.5	33.0
Model 17	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	32	11683.4	34.9
Model 18	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	28	11686.6	38.1
Model 19	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	24	11689.0	40.5
Model 20	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	23	11690.5	42.0
Model 21	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	32	11691.7	43.2
Model 22	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	26	11692.8	44.3
Model 23	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	25	11693.9	45.4
Model 24	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	30	11697.9	49.4
Model 25	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	29	11705.9	57.4
Model 26	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	30	11769.4	120.9
Model 27	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	29	11792.6	144.1
Model 28	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	19	11805.1	156.6
Model 29	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	19	11808.8	160.3
Model 30	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	26	11839.8	191.3
Model 31	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	17	11848.1	199.7
Model 32	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	15	11861.8	213.3
Model 33	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	15	11879.2	230.7
Model 34	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	33	11879.7	231.2
Model 35	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	13	11891.8	243.3
Model 36	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	22	11915.6	267.1
Model 37	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	24	11918.9	270.4
Model 38	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	23	11923.8	275.3
Model 39	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	21	11923.8	275.3
Model 40	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	21	11923.8	275.3
Model 41	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	27	11946.7	298.2
Model 42	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	27	11967.7	319.2
Model 43	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	25	11971.9	323.4
Model 44	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	23	12006.9	358.4
Model 45	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	16	12010.8	362.3
Model 46	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	14	12020.6	372.1
Model 47	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	14	12036.6	388.1
Model 48	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	7	not converged	

Table S5: Summary statistics for isofemale isolines. Percentages and sample sizes for mating and remating, and early fecundity of females paired with a control (23°C) or a heat-exposed (31°C) male. Note the smaller sample sizes for polyandry and fecundity due to low mating success of heat-exposed males.

Population	Isoline	Mating				Polyandry				4d Fecundity			
		23C	N	31C	N	23C	N	31C	N	23C	N	31C	N
Show Low	SLOB3	90%	41	42%	55	55%	31	94%	18	41.6±21.8	37	9.2±18.4	22
Show Low	2SLOC4	85%	41	47%	55	14%	28	91%	23	43.5±21.2	35	5.8±11.7	26
Show Low	SLOC48	90%	40	32%	56	52%	31	65%	17	49.1±20.2	35	27.1±27.2	18
Show Low	2SLOD29	85%	40	38%	55	15%	27	68%	19	48.3±17.9	34	16.2±27.2	21
Show Low	2SLOD33	85%	40	13%	56	57%	28	100%	7	46.5±18.9	34	0±0	7
Show Low	2SLOD6	83%	40	25%	55	41%	27	92%	13	47.5±16.8	33	16±20.9	14
Lewistown	LEW17	83%	40	37%	54	27%	30	71%	17	32.5±15.1	33	18.6±22.8	20
Lewistown	LEW23	88%	41	20%	55	57%	30	78%	9	42.2±16.1	36	15.6±21.2	11
Lewistown	LEW3	93%	40	18%	56	50%	30	100%	10	37.8±17.7	37	15.5±22.9	10
Lewistown	LEW64	83%	40	30%	56	46%	26	94%	16	37.3±20.9	33	20.1±24.9	17

Table S6: Model summary for polyandry (*cf* Table 3), additionally including copulatory behaviour from a female's first mating

binomial GLMM (N = 416)						
Fixed/Random effects	Coef	SE (Coef)	z	p	Var	SD
Intercept (control temperature)	-0.200	0.256	-0.78	0.437		
Heat-exposure	1.820	0.426	4.27	<0.001		
4d reproductive output (centred & scaled)	-0.393	0.167	-2.35	0.019		
First mate's size (centred & scaled)	-0.234	0.139	-1.68	0.093		
Female age (centred)	0.456	0.275	1.66	0.097		
Order in assay (centred & scaled)	0.090	0.147	0.61	0.540		
Log copulation latency (centred & scaled)	0.005	0.135	0.03	0.973		
Log copulation duration (centred & scaled)	0.074	0.136	0.55	0.585		
Heat:Reproductive_output	-0.518	0.347	-1.49	0.135		
Heat:First_mate_size	-0.437	0.302	-1.45	0.148		
<i>Male collection batch (16 levels)</i>					<0.001	<0.001
<i>Female isoline (10 levels)</i>					0.40	0.64
<i>Heat:Female_isoline (random slopes)</i>					0.21	0.46

Copulation latency and copulation duration were log-transformed and then scaled and centred to aid model convergence.

Supplementary figures:

Fig S1: Male heat-exposure affects multiple aspects of sexual behaviour. Courtship latencies (note the log-scale) of males that did not mate are shown as open circles. Bars illustrate approximate 95% confidence intervals, taken as twice the standard error calculated on the log-scale. Heat-exposed males (red) were less likely to court and mate, took longer to initiate courtship and to procure a mating, and mated for a shorter duration than control males (blue; see Tables 1 & S2).

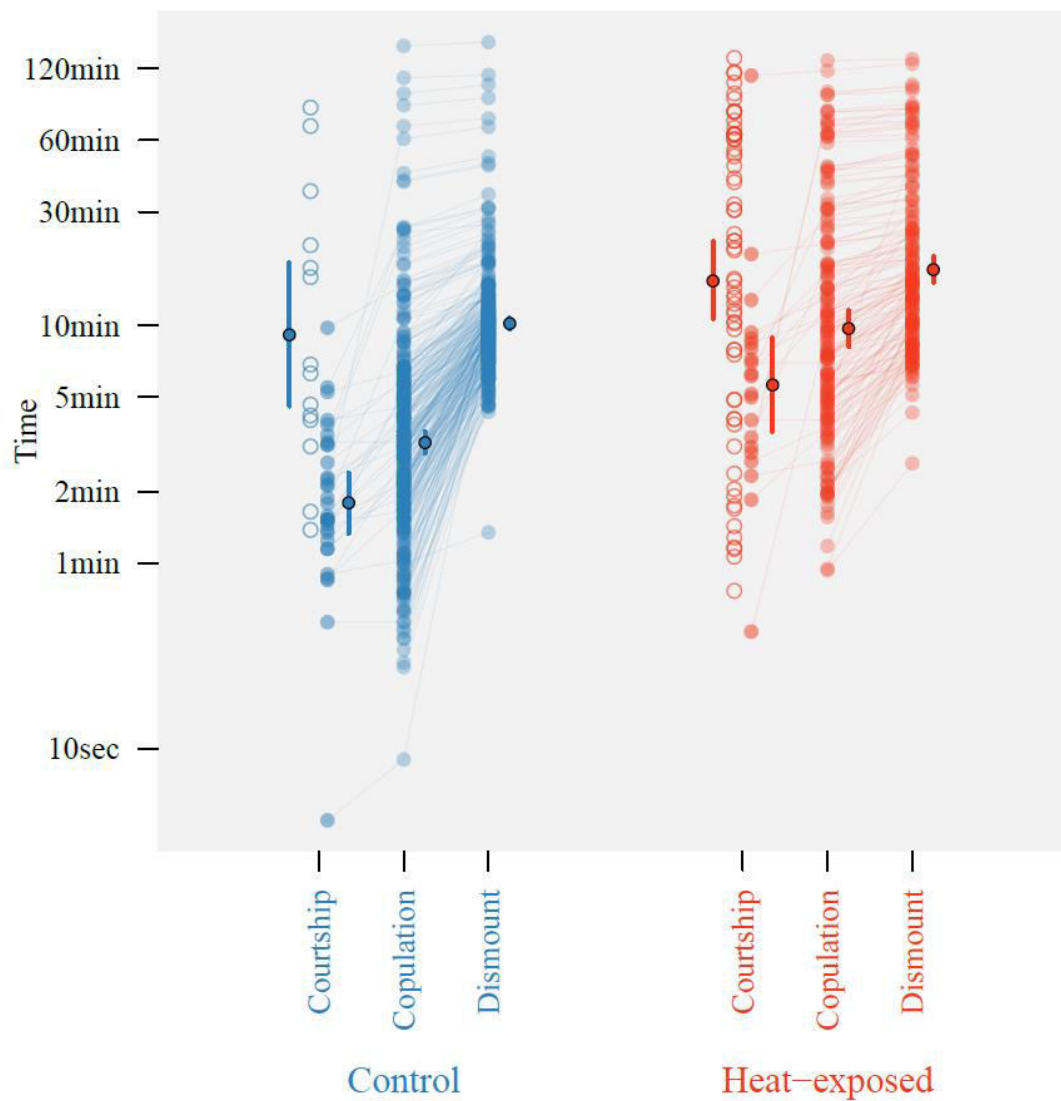


Fig S2: Lower reproductive output after the first mating is associated with increased polyandry. Ticks represent individual females, initially mated to heat-exposed (red) or control males (blue). Individual females are represented by ticks. Circles illustrate average polyandry for females within ranges of similar reproductive output (shaded horizontal bars), with surface area proportional to sample size. Irrespective of reproductive output, polyandry was higher after mating with heat-exposed males (main effect of male heat-exposure). The interaction between reproductive output and male heat-exposure was not significant (see Table 3) but is retained here for illustrative purposes.

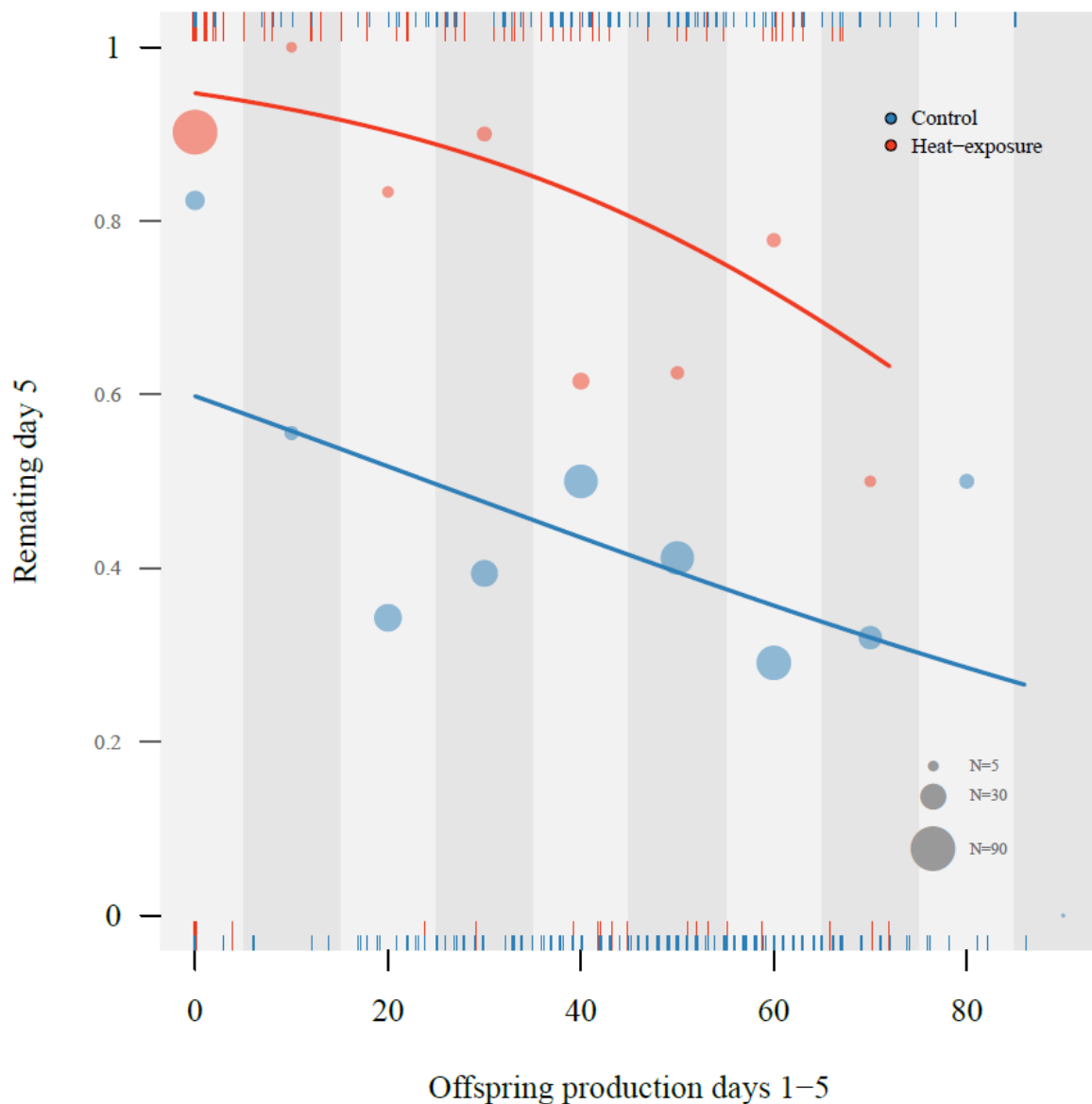


Fig S3: Heat-exposure decreased survival only in the male cohort A. Vials containing groups of up to 10 males were transferred into water baths set to 23°C (blue) or 31°C (red) one to two days (cohort A) or immediately (cohort B) after eclosion. Compared to survival (Table S2), mating performance was more similar for both male cohorts after heat-exposure (see Tables 1 & S1). Solid Bars illustrate approximate 95% confidence intervals and point surface area is proportional to the number of vials tested.

