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

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Data Accessibility Statement

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

Abstract

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

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

Introduction

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

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

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

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

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

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

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

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

Male heat-exposure treatment

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

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

Mating assays

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

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

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

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

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

Fitness consequences

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

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

Statistical analyses

To test the physiological impact of heat-exposure on males and its consequences for females we analysed the impact of heat-exposure on multiple aspects of male and 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.

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

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

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

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

Results

Heat-exposure reduces male survival and mating success

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

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

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

<u>Male heat-exposure reduces female reproductive fitness</u>

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

Polyandry restores female reproductive fitness in the face of male infertility

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

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

Discussion

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

Adaptively flexible polyandry

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

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

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

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

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

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

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

Conclusions

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

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726

Figure legends

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

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

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

Table 1: Summary statistics and sample sizes.

Temperature	Control (23°C)		Heat-exposure (31°C)				
Male cohort	A	B	A	B	Heat effect	Full model	Illustration
main experiment							
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
additional males							
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)	↓		

Given are mean, standard deviation and sample sizes for survival, mating behaviours and reproductive output. The effect of male heat-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.

	Conditional model						Zero-inflation model			
Fixed/*Random* effects	**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)

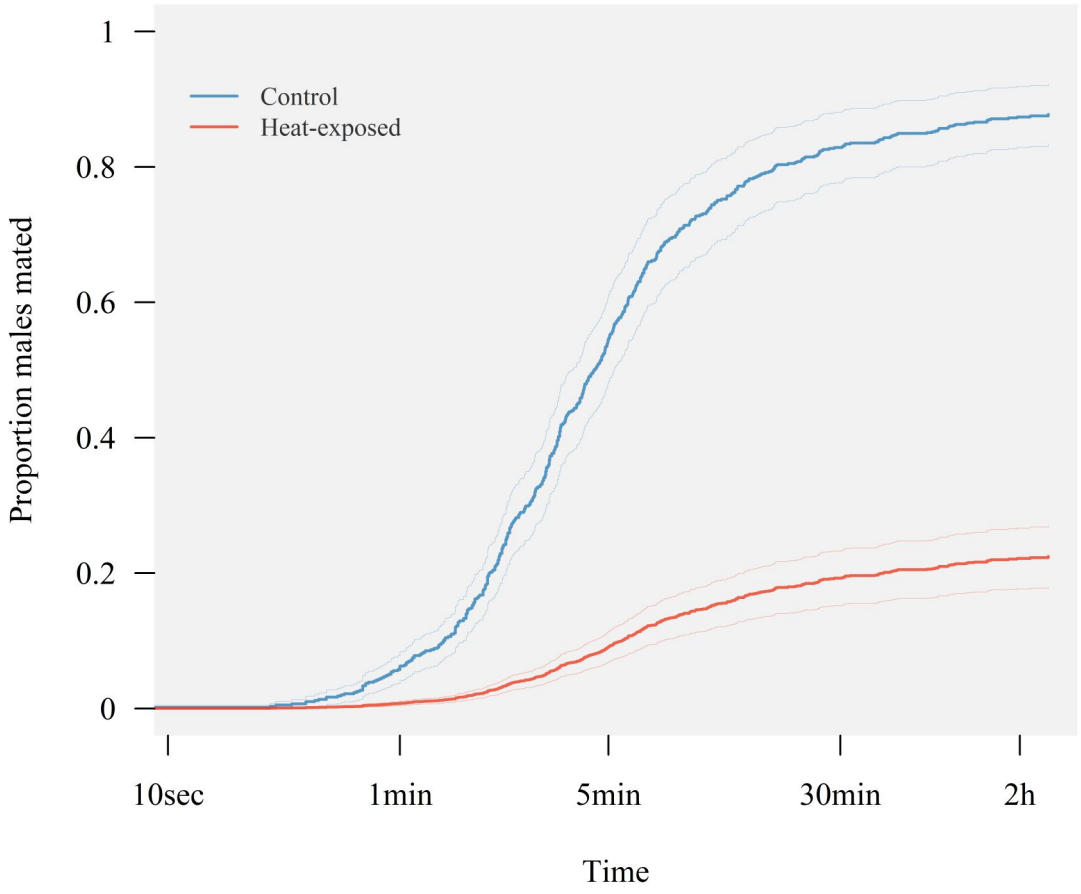
	Conditional model						Zero-inflation model			
Fixed/*Random* effects	**Coef**	**SE (Coef)**	**z**	**p**	**Var**	**SD**	**Coef**	**SE (Coef)**	**z**	**p**
Poly:Vial C	–6.831	3.914	–1.75	0.081			–3.287	0.850	–3.87	**<0.001**
Heat:Mono:Vial B	–32.379	12.860	–2.52	**0.012**						
Heat:Mono:Vial C	–21.605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

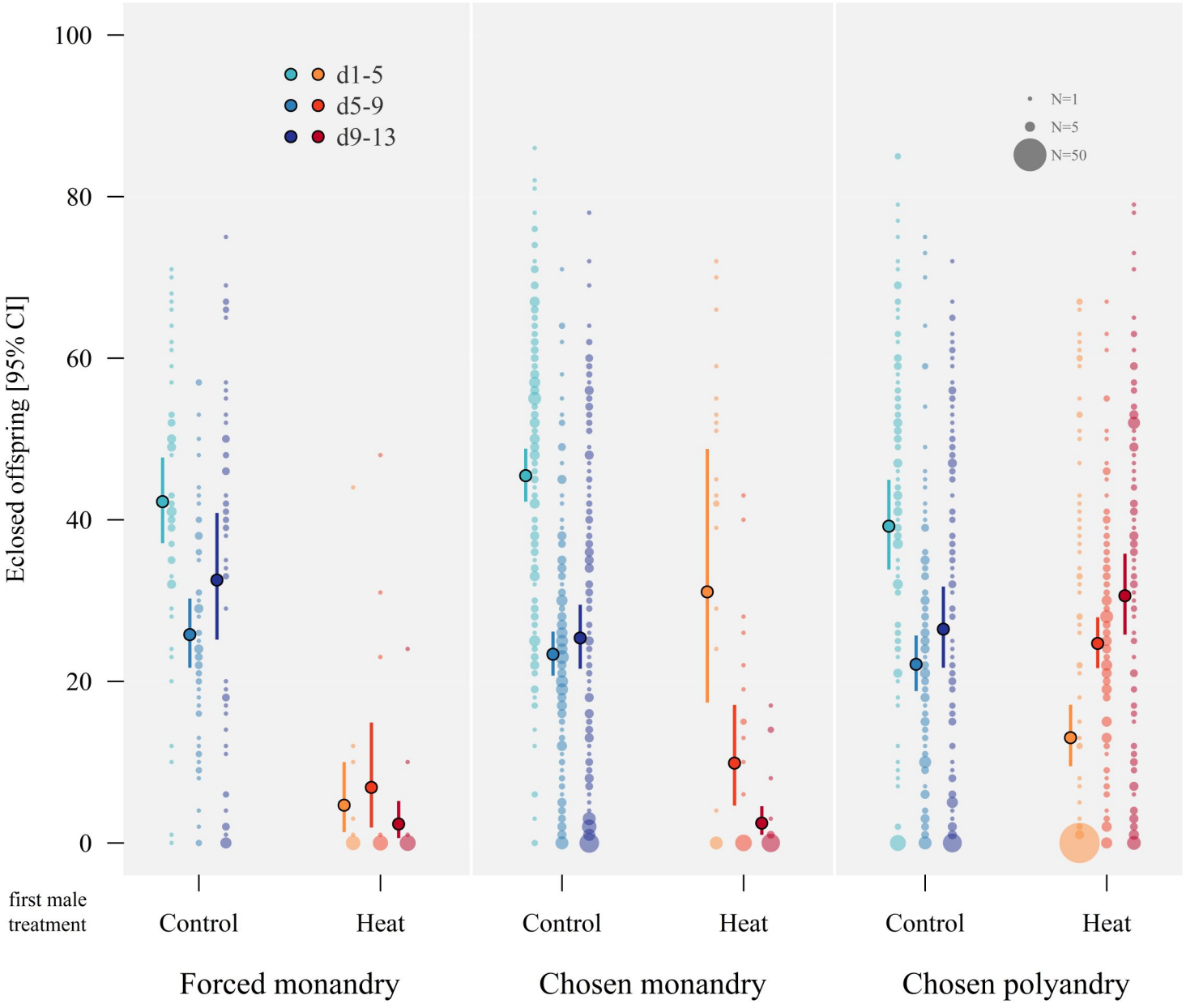
The conditional model describes the Gaussian component of female reproductive output (498 females) while the zero-inflation model accounts for the likelihood of reproductive failure. The model with the lowest AIC value was chosen as the best model. See Table S4 for an overview of models and associated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

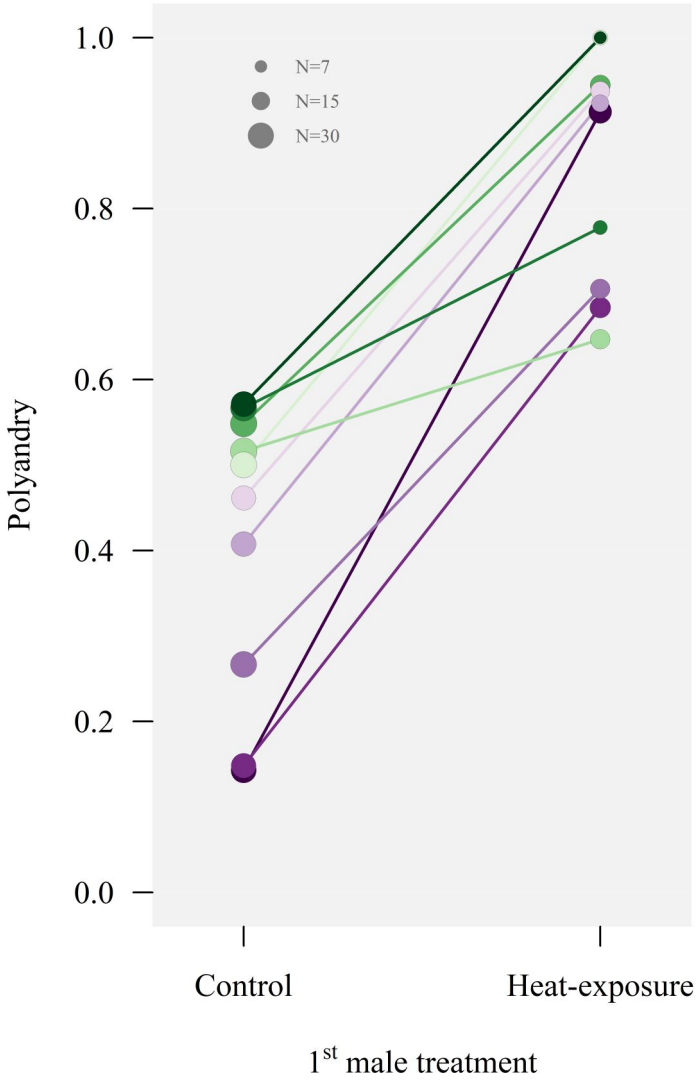
Table 3: Full model summary for polyandry.

	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		
Male collection batch (16 levels)					<0.001	<0.001
Female isoline (10 levels)					0.38	0.62
Heat:Female_isoline (random slopes)					0.21	0.46

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


Control
Heat-exposed
Proportion males mated
1
0.8
0.6
0.4
0.2
0
10sec
1min
5min
30min
2h
Time


100
80
60
40
20
0
Eclosed offspring [95% CI]
d1-5
d5-9
d9-13
N=1
N=5
N=50
first male treatment
Control
Heat
Control
Heat
Control
Heat
Forced monandry
Chosen monandry
Chosen polyandry


1.0
0.8
0.6
0.4
0.2
0.0
Polyandry
N=7
N=15
N=30
Control
Heat-exposure
1st male treatment

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

	Mating success (binomial GLMM; N = 916)						Copulation latency (log LMM; N = 496)						Copulation duration (log LMM; N = 487)					
Fixed/*Random* effects	**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

	Courtship latency (log LMM; N = 127)						Courtship duration (log LMM; N = 52)					
Fixed/*Random* effects	**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**	-	-
Housing vial (155 levels)	-	-	-	-	1.51	1.23
Population (4 levels)	-	-	-	-	<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	-	-
Virgin collection batch (16 levels)	-	-	-	-	-	0.003	0.053
Residual	-	-	-	-	-	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											
	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	df	AIC	ΔAIC
Model 1	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		40	11650.1	1.6
Model 3	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	42	11654.6	6.1
Model 5	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		34	11664.5	16.0
Model 7	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	38	11668.7	20.2
Model 9	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	36	11672.3	23.8
Model 11	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	36	11676.2	27.7
Model 13	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		28	11678.7	30.2
Model 15	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	34	11681.5	33.0
Model 17	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		28	11686.6	38.1
Model 19	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		23	11690.5	42.0
Model 21	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		26	11692.8	44.3
Model 23	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	30	11697.9	49.4
Model 25	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			30	11769.4	120.9
Model 27	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				19	11805.1	156.6
Model 29	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					26	11839.8	191.3
Model 31	x	x	x		x						x	x	x	x						17	11848.1	199.7
Model 32	x	x	x		x						x	x		x						15	11861.8	213.3
Model 33	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		33	11879.7	231.2
Model 35	x	x	x		x						x	x								13	11891.8	243.3
Model 36	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		24	11918.9	270.4
Model 38	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		21	11923.8	275.3
Model 40	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					27	11946.7	298.2
Model 42	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					25	11971.9	323.4
Model 44	x	x	x		x	x	x	x		x	x									23	12006.9	358.4
Model 45	x	x	x		x						x		x	x						16	12010.8	362.3
Model 46	x	x	x		x						x			x						14	12020.6	372.1
Model 47	x	x	x		x						x		x							14	12036.6	388.1
Model 48	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.

		Mating				Polyandry				4d Fecundity			
Population	**Isoline**	**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		
Male collection batch (16 levels)					<0.001	<0.001
Female isoline (10 levels)					0.40	0.64
Heat:Female_isoline (random slopes)					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).

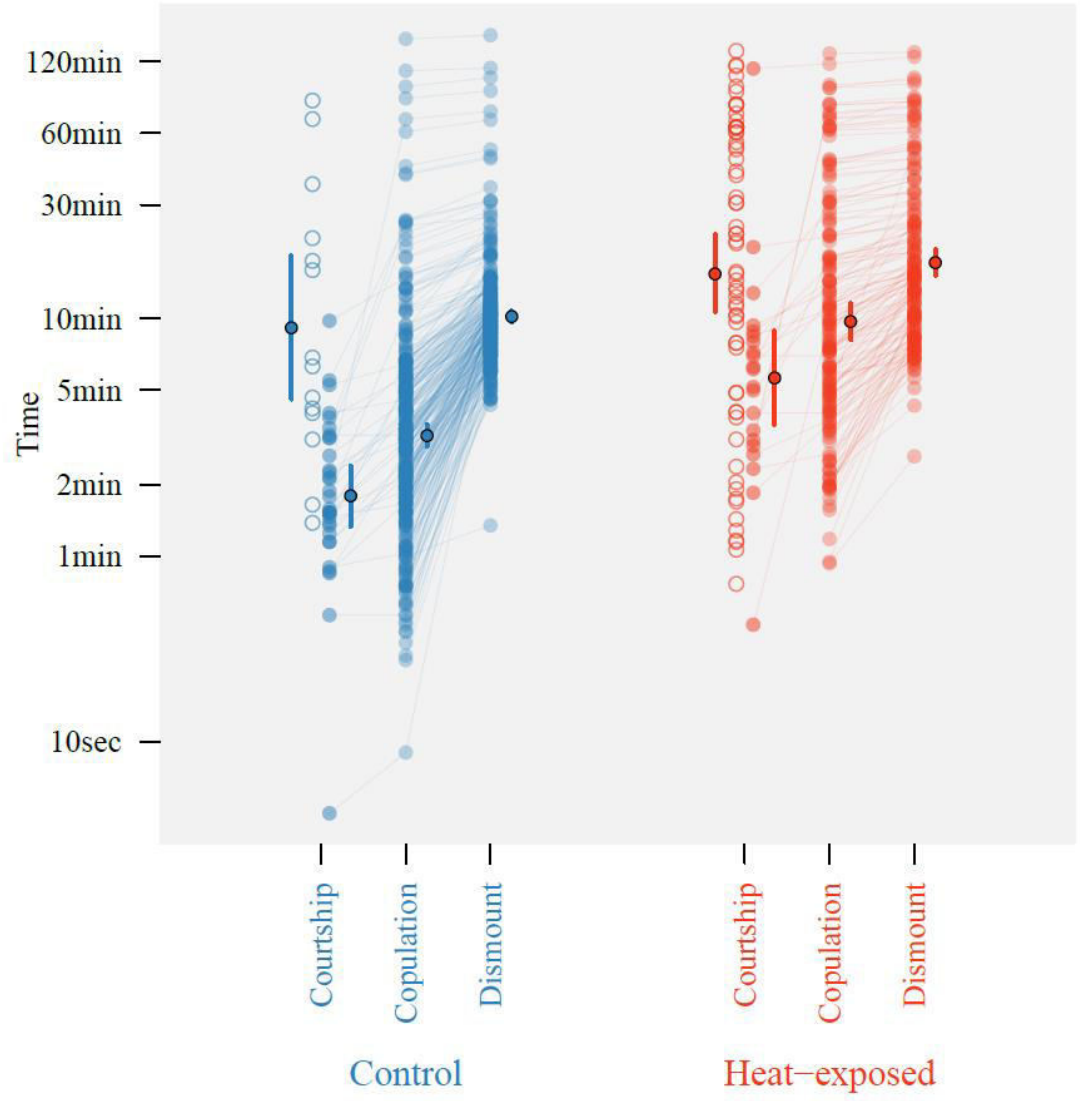

Fig S2: Lower reproductive output after the first mating is associated with increased polyandry. Ticks represent individual females, initially mated to heat-exposed (red) or control males (blue). Individual females are represented by ticks. Circles illustrate average polyandry for females within ranges 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.

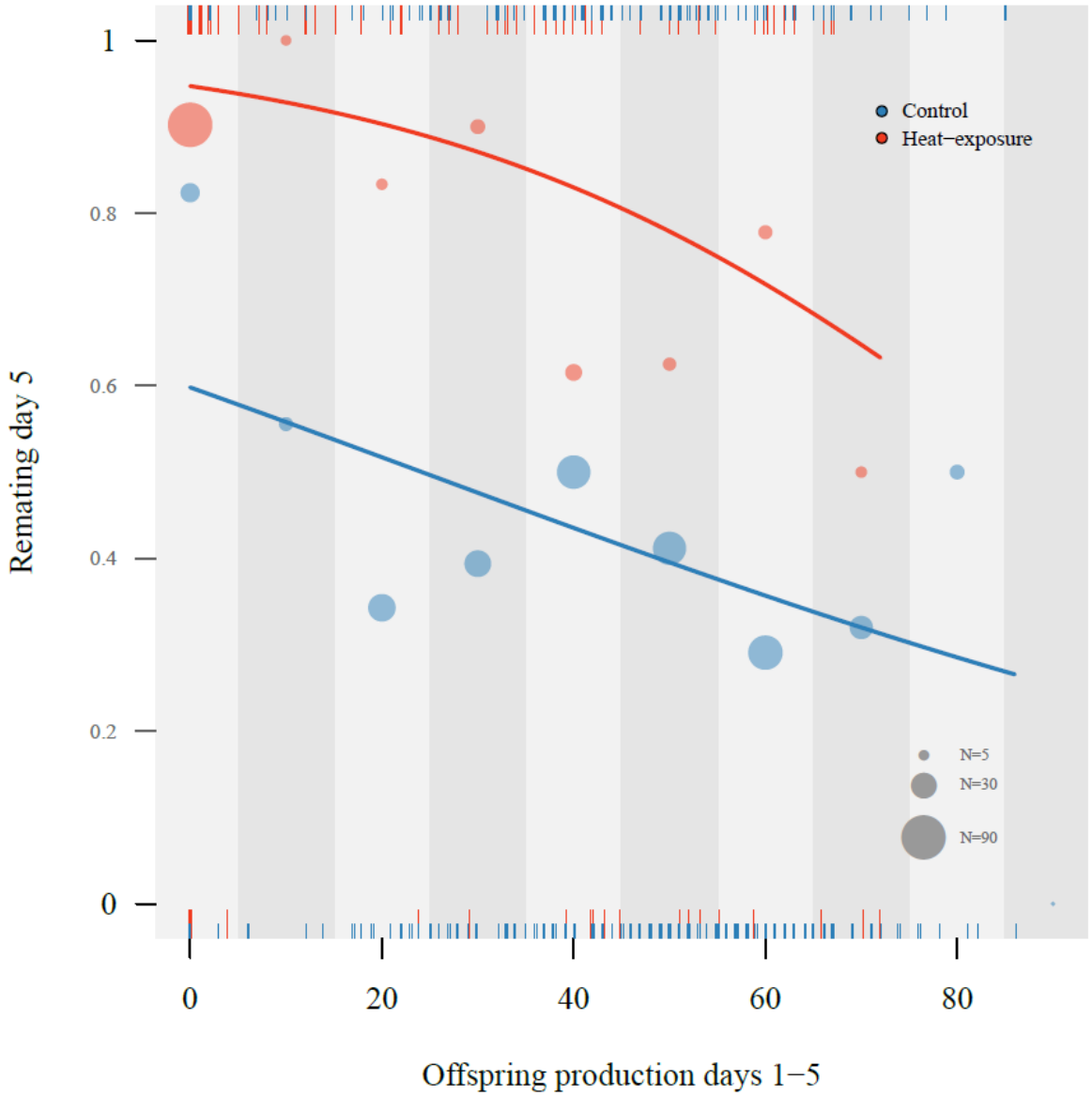

Fig S3: Heat-exposure decreased survival only in the male cohort A. Vials containing groups of up to 10 males were transferred into water baths set to 23°C (blue) or 31°C (red) one to two days (cohort A) or immediately (cohort B) after eclosion. Compared to survival (Table S2), mating performance was 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.