

1 **A me Temperature drives the evolutionary diversification of male harm in *Drosophila***
2 ***melanogaster* flies**

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26

27 **ABSTRACT**

28 Sexual selection often leads to sexual conflict via pre-copulatory (harassment) and/or
29 copulatory (traumatic insemination) male harm to females, impacting population growth,
30 adaptation and evolutionary rescue. Male harm mechanisms are diverse and taxonomically
31 widespread, but we largely ignore what ecological factors modulate their diversification.
32 Here, we conducted experimental evolution under cold ($20\pm 4^\circ\text{C}$), moderate ($24\pm 4^\circ\text{C}$) and hot
33 ($28\pm 4^\circ\text{C}$) thermal regimes in *Drosophila melanogaster*, a species with male harm via
34 harassment and seminal fluid proteins (SFPs), to show that temperature drives the divergent
35 evolution of sexual conflict. At the cold regime, evolution resulted in reduced and less plastic
36 harassment (i.e. pre-copulatory harm) while, at the hot regime, it was characterized by
37 responses in the seminal proteome driven by differential expression of SFPs. Our results
38 suggest that temperature can be key to understand the past diversification and future (global
39 warming) evolution of sexual conflict, and the maintenance of genetic variation in male harm
40 traits.

41

42 **Introduction**

43 Sexual selection can improve population viability and evolvability, making populations better
44 able to adapt to a changing environment (Cally et al., 2019; Lorch et al., 2003; Lumley et al.,
45 2015; Martinez-Ruiz & Robert, 2017; Martinossi-Allibert et al., 2019). Driven by
46 competition for mates and their gametes, sexual selection is widespread and important in both
47 females and males (Fromonteil et al., 2023). Nevertheless, anisogamy commonly results in
48 asymmetries in the strength and form of sexual selection across the sexes (Janicke et al.,
49 2016). Typically, stronger sexual selection in males allows for the effective purging of
50 deleterious mutations and the capture of good genes (condition-dependent genic capture) at a
51 relatively cheap demographic cost, inasmuch females are spared the brunt of selection
52 (Agrawal, 2001; Whitlock & Agrawal, 2009). However, the same divergent selective
53 pressures that make sexual selection such an effective evolutionary sieve also set the scene
54 for sexual conflict, where female and male evolutionary interests misalign (Parker, 1979).
55 Alleles that confer a reproductive advantage to one sex may have opposing effects in the
56 other, leading to reproductive strategies that evolve against each other (Pizzari & Snook,
57 2003). Such sexually antagonistic coevolution is particularly salient in polygamous species,
58 where it frequently leads to adaptations in males that make them better competitors in the
59 sexual selection arena, but at the expense of harming females (Arnqvist & Rowe, 2005).

60 Harmful male adaptations to females (male harm) are pervasive, diverse and
61 sophisticated across the tree of life. On the one hand, male harassment of females during pre-
62 copulatory competition for mating has been documented in many vertebrate and invertebrate
63 species (Gómez-Llano et al., 2024). On the other, post-copulatory competition has given rise
64 to a variety of male harm adaptations that are similarly widespread, ranging from potentially
65 harmful ejaculates (Wigby & Chapman, 2005) to adaptations for traumatic insemination
66 (Crudgington & Siva-Jothy, 2000). Male harm thus drives antagonistic female-male co-

67 evolution in a host of behavioural and morphological traits (Arnqvist & Rowe, 2005), and
68 may even act as an engine of speciation (Gavrilets, 2014; Rice et al., 2005). More
69 importantly, male harm can impact population demography by depressing net female
70 productivity (Gómez-Llano et al., 2024), even to the point of facilitating extinction (Le
71 Galliard et al., 2005). Recent theoretical models suggest that such negative effects may
72 compound when harmful traits are linked to condition (Flintham et al., 2023; Gómez-Llano et
73 al., 2024; Pitnick & García-González, 2002). In short, sexual selection acts as a double-edge
74 sword for populations because stronger condition-dependent selection on males, which
75 allows for the demographically cheap purging of deleterious alleles, the genic capture of
76 good genes, and ensuing fast adaptation, is also a recipe for intense sexual conflict.
77 Understanding what factors determine whether strong sexual selection and resulting conflict
78 leads to harm to females, and the nature and diversity of its underlying traits, is a central
79 question in evolutionary biology.

80 A growing body of research highlights ecology as a crucial factor for understanding
81 the evolution of male harm and its consequences for populations (Perry & Rowe, 2018).
82 Ecology has been shown to play a central role in shaping patterns of population divergence
83 via sexual conflict (Arbuthnott et al., 2014; Perry et al., 2017), as well as in determining the
84 intensity of male harm and to what degree it may offset the advantages of good-genes
85 selection (Londoño-Nieto et al., 2023; Yun et al., 2017,2018). Recent studies show that
86 environmental factors such as spatial complexity (Berger & Liljestränd-Rönn, 2024;
87 MacPherson et al., 2018; Malek & Long, 2019), nutritional status (Fricke et al., 2010), or sex
88 ratio and population density (Chapman et al., 2003; Gomez-Llano et al., 2018), have the
89 potential to modulate male harm via both plastic and evolutionary responses. However,
90 while such evidence suggests that male harm seems to be generally higher in environments to

91 which populations are adapted to, we largely ignore the degree to which ecological effects are
92 predictable across species.

93 Temperature is a particularly interesting ecological factor to this respect. It modulates
94 a wide range of phenotypic traits, impacting individuals and populations at a global
95 taxonomic scale, and exhibits marked spatio-temporal variation such that, for most species in
96 the wild, competition for reproduction (and consequently male harm) will unfold in a
97 dynamic thermal environment. This is being taken to the extreme by global warming.
98 Furthermore, thermodynamics dictate that temperature imposes similar adaptive challenges
99 across species, particularly in ectotherms. For example, protein stability and sperm
100 production and function seem to be particularly sensitive to hot temperatures, while cold
101 temperatures pose general constraints on behaviour and activity (Berger et al. 2021;
102 Dougherty et al. 2024). Importantly, recent research in *Drosophila melanogaster* shows that
103 the intensity of male harm, its impact on female fitness components, and its underling
104 mechanisms are very thermally plastic (Londoño-Nieto et al., 2023). During male-male pre-
105 copulatory competition, males harm females via intense harassment that causes substantial
106 costs in the form of physical injuries and energetic and opportunity costs (Bretman & Fricke,
107 2019; Partridge & Fowler, 1990; Teseo et al., 2016), but male harassment and its impact on
108 females is drastically reduced when exposed to cold temperatures (Londoño-Nieto et al.
109 2023). In the context of sperm competition, some male seminal fluid proteins (SFPs) affect
110 female re-mating and egg-laying rates to the male's advantage, but this can come at a cost to
111 female fitness (Chapman, Bangham, et al., 2003; Hopkins & Perry, 2022; Wigby &
112 Chapman, 2005). SFPs are secreted by male accessory glands and are strategically allocated
113 by males in response to even subtle variations in the socio-sexual context (Hopkins et al.,
114 2019a,b; Sirot et al., 2011), but hot temperatures seem to curtail their impact on female

115 reproduction (Londoño-Nieto et al. 2023). These findings suggest that temperature may be
116 key to understand the evolution and diversification of male harm (García-Roa et al., 2020).

117 Here, we test this idea by addressing whether adaptation to different temperatures
118 results in the evolution of higher male harm in adapted vs. maladapted temperatures and,
119 more importantly, whether pre-copulatory (behavioural, activity related) vs. copulatory
120 (seminal fluid proteins) mechanism respond differently to cold vs. hot thermal regimes. To
121 this aim, we collected *D. melanogaster* from a population that has been shown to be
122 thermally plastic for male harm (Londoño-Nieto et al., 2023) and set up 12 experimental
123 evolution populations under three different thermal regimes mimicking natural seasonal and
124 circadian temperature variation. After 29-30 generations of experimental evolution, we set up
125 a series of fitness, behavioural and seminal proteome assays to measure experimental
126 evolution effects on: male harm intensity (i.e. how much male-male competition depresses
127 female fitness), the thermal plasticity of such effects, and underlying pre- (male aggression
128 and harassment levels) and copulatory (SFPs) traits.

129

130 **Methods**

131 Experimental evolution design

132 We established 12 populations from our field-collected population “Vegalibre” (see
133 Londoño-Nieto et al., 2023), and subjected them to experimental evolution under one of three
134 temperature regimes: average of 20, 24 or 28°C with daily pre-programmed fluctuations of
135 $\pm 4^\circ\text{C}$ mimicking circadian temperature variation (Fig. S1), at ~60% humidity, a 12:12hr
136 light:dark cycle, and in non-overlapping generations, controlling for population size and
137 density. Four populations (replicates) evolved at each temperature regime: populations A-D
138 at $20\pm 4^\circ\text{C}$ (*cold*); populations E-H at $24\pm 4^\circ\text{C}$ (*moderate*), and populations I-L at $28\pm 4^\circ\text{C}$
139 (*hot*). Each generation began by releasing 100 males and 100 females (N=200), randomly

140 selected and of the same age, into a glass jar (16.5x19.5cm) containing two bottles with
141 standard food. We allowed 6 days of interaction, collecting eggs on the 6th day that we raised
142 at a standardized density (Clancy & Kennington, 2001) in bottles with standard food. We
143 isolated emerging virgins from these bottles in same-sex vials and used them to setup the next
144 generation when 3-4d old. This design selected for early reproduction, ignoring the
145 cumulative harm effects over time that are typical of male harm and thus minimising
146 selection for female resistance (Bonduriansky et al., 2008; Filice et al., 2020). Populations
147 were assayed after 29-30 generations of experimental evolution and two generations of
148 common garden at 24±4°C to control for parental and grand-parental effects. Environmental
149 conditions across all assays were controlled meticulously to ensure common garden
150 conditions (see SI for full details).

151 Male harm and behavioural assays (experiment 1)

152 To examine the effect of thermal evolution regimes on overall male harm levels and its
153 thermal plasticity, we compared reproductive success and survival of female flies from each
154 population under monogamy (low sexual conflict; one female and one male per vial) and
155 polygamy (high sexual conflict; one female and three males per vial). This is standard
156 procedure to gauge male harm in *Drosophila*, where these sex ratios represent biologically
157 relevant scenarios (Dukas, 2020; Yun et al., 2021). For each population within each
158 experimental evolution regime, we replicated these assays at 20, 24, and 28°C. We collected
159 experimental flies as virgins, isolated them into same-sex vials of 15 individuals and then
160 randomly allocated them to either of the three temperature treatments 48h before starting the
161 experiment, at which temperature they remained until the end of assays. We began
162 experiments by placing virgin focal females (4-5d old) in individual vials containing medium
163 with live yeast, after which we immediately added one (monogamy) or three (polygamy)
164 experimental males from the corresponding population. On day 1 of the experiment, we

165 observed flies (Table 1) for 8h combining scan sampling with an all-occurrences recording
166 rule (see SI) to score courtship intensity (courting males per female per hour), male-male
167 aggression rate (aggressions per hour) and female rejection rate (rejections per hour; Bastock
168 & Manning, 1955) to investigate whether pre-copulatory male harm mechanisms, and their
169 thermal plasticity, were affected by experimental evolution. To estimate female reproductive
170 success, we transferred flies to fresh vials twice a week and incubated vials containing eggs
171 at $24\pm 4^{\circ}\text{C}$ for 15-20d to allow F1 offspring emergence, and froze them for later counting.
172 Differences in incubation time are due to differences in developmental temperature during
173 the first 1-4 days (depending on when individual eggs were laid in relation to when vials
174 were flipped). We discarded and replaced males with young (2-4d old) virgin males (same
175 treatment as described above) three weeks after starting the experiment. We kept flies under
176 these conditions for six weeks, during which time we recorded survivorship of focal females
177 daily and replaced dead male flies with stock males maintained at each of the temperature
178 treatments. Samples sizes for female reproductive success and survivorship are in the Table
179 1.

180 To explore male harm, we modelled reproductive success (sum of offspring for a
181 female across the six weeks) as the response variable in a linear mixed model (LMM), and
182 courtship, male-male aggression and female rejection rates as the response variables in
183 generalized linear mixed models (GLMM) with experimental evolution regime, temperature
184 treatment, mating system and their interactions as fixed effects, and replicate population as a
185 random effect using *lme4* (Bates et al., 2015) and *glmmTMB* (Brooks et al., 2017) packages
186 in RStudio. We modelled survivorship as the response variable in a Cox proportional-hazard
187 model with the same fixed and random effects using *coxme* and *survminer* packages
188 (Kassambara & Kosinski, 2018; Therneau, 2022). Additionally, to further explore if overall
189 harm was higher at adaptive vs. maladaptive temperatures, we performed a complementary

190 analysis where we modelled experimental evolution regime, mating system and adaptive
191 temperature (yes/no, according to whether temperature was inside/outside of experimental
192 evolution regime) and the interaction between mating system and adaptive temperature as
193 fixed effects, and replicate population as random effect. In this analysis, we only used data
194 from populations evolved at the cold (for which 28°C is maladaptive) and hot (for which
195 20°C is a maladaptive) regimes; for flies evolved at the moderate regime all temperatures
196 were inside its experimental evolution regime.

197 For behavioural data, previous studies in this species have shown that pre-copulatory
198 male harm is linked to courtship intensity, female rejection and male-male aggression (i.e.
199 male intrasexual competition; Carazo et al., 2014; Partridge & Fowler, 1990). Following the
200 approach used in previous studies (e.g. Carazo et al., 2014), Component 1 of a PCA
201 explained 67.7% of variation in behavioural data, whereby male-male aggression, courtship
202 intensity and female rejection all loaded in the same direction (Table S2a), so we took PC1 as
203 a combined measure of male-male competition and courtship/harassment to females. Note,
204 however, that we also modelled these behaviours separately with very similar results (see SI).

205 In all cases, when we detected a significant interaction between main effects, we ran
206 models separately for each evolutionary temperature regime or temperature treatment and run
207 post hoc Tukey's test to explore the nature of such interactions. We assessed significance
208 with *F* test for LMM and chisquare test for GLMM and Cox proportional-hazard models. For
209 further analysis details see SI.

210 Proteomics assays (experiment 2)

211 To study whether and how the seminal proteome of males that are competing for females for
212 the first and successive matings evolves in response to temperature, we set up a series of
213 assays and conducted label-free quantitative proteome analysis of the accessory glands of

214 virgin (first mating) and mated (successive matings) males across experimentally evolved
215 regimes. We conducted assays at the common garden temperature of 24°C (shared
216 temperature across thermal regimes). Upon eclosion, we allocated virgin focal males into
217 vials of 8 individuals until 4-5d-old. On the day of sample collection, we isolated 45
218 experimental females per population in vials, after which we immediately introduced focal
219 males either into a female-containing (mated) or empty (virgin) vial. We flash-froze mated
220 males in liquid nitrogen 25min after the start of mating, freezing a virgin male from the same
221 population at the same time (see Hopkins et al., 2019a,b; Sepil et al., 2019). We repeated this
222 procedure during two more consecutive days to obtain three independent biological replicates
223 following a balanced design. We stored all frozen samples at -80°C until dissection, for
224 which we thawed flash frozen males and dissected their accessory glands on ice in
225 phosphate-buffered saline (PBS) buffer, under a Leica M80 binocular scope. Each biological
226 replicate (i.e. sample) consisted in a pool of 20 reproductive glands from males of the same
227 temperature regime, mating status and replicate, which we sent for label-free quantitative
228 proteomics sample preparation and quantification (protocol SWATH-MS; Gillet et al., 2012)
229 at the SCISIE (University of Valencia) proteomics service (see SI for details). We thus
230 analysed six samples per population (three virgin and three mated males), across four
231 replicate populations per experimental evolution regime (i.e. $n = 72$). All assayed males
232 across experimental evolution regimes were dissected at the same time (i.e. in balanced order
233 across the same days) and proteomic analyses conducted at the same time for all samples, to
234 avoid block effects.

235 We conducted all proteomics analysis on normalized abundances (see SI), generating
236 two different proteomics data sets (i.e. virgin and mated males). We used an elastic net
237 regression model and tests of reduction of dimensionality PLS-DA to analyse our data sets,
238 using *glmnet* (Friedman et al., 2010) and *mixOmics* (Rohart et al., 2017) packages in RStudio.

239 For our analysis and visualization of abundance patterns we averaged across biological
240 replicates for each protein, population, experimental evolution regime and mating status. For
241 visualization, we used a Euclidean correlation distance metric and plotted the output as a
242 heatmap using *NMF* package (Gaujoux & Seoighe, 2010). We identified seminal fluid
243 proteins (SFPs) based on a high-confidence SFPs reference list from Sepil et al., (2019) and
244 Wigby et al., (2020). We represented Venn diagrams on the number of proteins and
245 percentage of SFPs using *ggvenn* package (Gao et al., 2021). Finally, to study differential
246 evolution of SFPs, we repeated the elastic net regressions including only the SFPs dataset.

247 GxE assay (experiment 3)

248 To test for GxE interactions in male fitness within the range of temperatures at which
249 reproduction is optimal for the ancestral wild population (20-28°C) of our focal flies, we
250 conducted a series of fitness assays across 30 male genotypes (i.e. isogenic lines) derived
251 from wild-caught flies from this wild population. We established isolines through 10
252 generations of inbreeding, resulting in flies sharing at least 96% of their genome (Falconer,
253 1996). Before the start of the experiment, we isolated 40 females per isoline into embryo egg-
254 laying cages with yeasted grape juice agar plates, from which we collected experimental
255 virgin wild-type (*wt*) male flies that we placed into same-sex vials of 15 individuals. We used
256 *sparkling^{poliert}* (*spa^{pol}*) backcrossed into the Vegalibre population (i.e. same genetic
257 background) as rival males and reproductive females, a recessive phenotypic marker that can
258 be used for paternity assignment. To begin the experiment, we placed *wt* males from each
259 isoline in individual vials with medium, after which we added two *spa^{pol}* males and one
260 female (high-competition environment). We then placed four replicates (i.e. vials) per isoline
261 under three different treatment temperatures (20, 24, and 28°C, $\pm 4^\circ\text{C}$). We did not have
262 enough flies to set up four replicates in 6 isolines (see Data), so we ended up with 114
263 replicates per temperature treatment. We replaced *spa^{pol}* females every two weeks and *spa^{pol}*

264 males every four weeks, so that focal males competed over access to different females against
265 different males during their lifespan, as happens in nature. We recorded survivorship and
266 offspring production as described for experiment 1. We calculated reproductive success of
267 focal males as the proportion of sired offspring vs. total offspring ($wt + spa^{pol}$), and modelled
268 it as the response variable in a GLMM using a Beta regression model (Smithson &
269 Verkuilen, 2006), with temperature as a fixed effect and isoline and their interaction as
270 random effects (Bolker et al., 2009) using *glmmTMB* (Brooks et al., 2017) package in
271 RStudio.

272 **Results**

273 *Harm is higher at evolved temperatures*

274 Experimental evolution significantly modulated the degree to which high conflict hampered
275 female reproductive success, with higher male harm in flies from the moderate regime
276 (experimental evolution regime x mating system interaction: $F_{2,2939.1} = 3.04$, $P = 0.048$; Figs.
277 1 and S2). This was driven mainly by the fact that male harm was more constant (less plastic)
278 in flies evolved in the moderate thermal regime, whereas it was lower at 28°C in flies evolved
279 in the cold thermal regime and at 20°C in flies evolved in the hot thermal regime (i.e. their
280 respective non-adapted temperatures; experimental evolution regime x temperature treatment
281 interaction: $F_{4,2939.5} = 2.89$, $P = 0.021$; Fig. 1, Table S3). Effects on female survival closely
282 mimicked effects on female reproductive success (experimental evolution regime x mating
283 system interaction: $X^2_2 = 8.30$, $P = 0.016$; experimental evolution regime x temperature
284 treatment interaction: $X^2_4 = 55.92$, $P < 0.001$; Fig. S3, Table S4a,b). Finally, direct
285 comparison of male harm levels between adapted vs. non-adapted temperatures confirmed
286 that, as predicted, overall male harm was higher at temperatures within vs. outside the
287 thermal range at which flies had evolved (adaptive temperature x mating system interaction

288 for female reproductive success, $F_{1,1942.1} = 4.12$, $P = 0.042$, and survival, $X^2_1 = 10.89$, $P <$
289 0.001 ; see Figs. 1 and S3).

290 *Male harassment decreases at colder temperatures*

291 Experimental evolution regime had clear effects on both overall male harassment and its
292 plasticity. Overall, harassment was lower ($F_{2,9} = 3.87$, $P = 0.06$) and less plastic
293 (experimental evolution x temperature treatment: $F_{4,1039.1} = 2.95$, $P = 0.019$; Fig. 2, Table
294 S2b,c) in flies evolved in the cold thermal regime. Analysing these three behaviours
295 separately, as well as effects across mating systems, confirmed these results (see SI).

296 *The male seminal proteome diversifies across thermal regimes, with SFPs characterising* 297 *evolution at hot temperatures*

298 In experiment 2, we found a total of 1453 proteins, 149 priorly identified as SFPs. For virgin
299 males, 37 proteins were selected as predictor variables with a strong effect on proteome
300 quantification, 8 of which are known SFPs. Euclidean distance correlation identified three
301 different clusters for these 37 proteins, which coincide with the three experimental evolution
302 thermal regimes (Fig. 3a). A partial least-squares discriminant analysis (PLS-DA) supported
303 these findings (Fig. 3b). We identified 5 and 16 proteins that were singularly over and under-
304 expressed, respectively, by males that evolved in the hot regime, eight and two that were over
305 and under-expressed by males that evolved in the moderate regime, and four and two that
306 were singularly over and under-expressed by males that evolved in the cold regime (Fig.3c).
307 While 80% of the proteins differentially over-expressed in flies from the hot regime have
308 been previously identified as SFPs, only one of the differentially overexpressed proteins at
309 the moderate regime, and none at the cold regime, are known SFPs (Fig. 3c, Table S5). We
310 found over-expression of the SFP “Semp1” by males evolved in the hot regimen. This protein
311 has been described to be transferred to females during mating and is necessary to process the

312 ovulation hormone ovulin and the sperm storage protein Acp36DE in mated females
313 (LaFlamme et al., 2014; Ravi Ram et al., 2006).

314 Results from mated males closely resembled the above results. Elastic net regression
315 identified 129 proteins as predictor variables with a strong effect on proteome quantification
316 in mated males, 24 previously identified as SFPs. According to the abundance of those
317 proteins, we again identified three different clusters that coincide with the three experimental
318 evolution regimes (Fig. 4a), confirmed by the PLS-DA analysis (Fig. 4b). 16 and 46 proteins
319 were differentially over and under-expressed, respectively, by males evolved in the hot
320 regime. 14 and 13 were differentially over and under-expressed by males evolved in the
321 moderate regime, and 13 and 27 were differentially over and under-expressed by males
322 evolved in the cold regime. 44% of the proteins over-expressed by males evolved in the hot
323 regime are known SFPs, while 7,4% and 7,6% of the proteins differentially over-expressed at
324 moderate and cold thermal regimes, respectively, are known SFPs (Fig 4c, Table S5). We
325 found higher expression of the ovulation hormone (ovulin) by males evolved in the hot
326 regime and higher expression of Acp70A by males evolved in the cold regime.

327 Finally, focusing exclusively on the 149 proteins previously identified as SFPs, we
328 found that, for both virgin and mated males, clusters consistently aligned with the thermal
329 regimes. Elastic net regression identified 56 and 85 proteins as predictor variables with a
330 strong effect on SFPs quantification in virgin and mated males, respectively. Importantly,
331 SFPs responsible for inducing physiological and behavioural changes in mated females were
332 predominantly over-expressed by males evolved in the hot regime, regardless of mating
333 status (Fig. 5).

334 *Strong thermal GxE in male reproductive success of the ancestral wild population*

335 For experiment 3, we found clear thermal GxE interactions for male reproductive success
336 ($X^2_{10} = 4.26$, $P < 0.001$). The two most common patterns of response (reaction norms)
337 showed male genotypes that either had higher reproductive success at moderate vs. hot and
338 cold temperatures (negative quadratic pattern) or higher reproductive success at hot and cold
339 vs. moderate temperatures (positive quadratic pattern; see Fig. S4).

340 **Discussion**

341 Here, we combined experimental evolution with behavioural, fitness and proteomic assays in
342 *Drosophila melanogaster* originating from a wild population to show that thermal ecology
343 can drive the evolution and diversification of male pre-copulatory and copulatory sexual
344 conflict traits and resulting male harm to females. Our results suggest that temperature might
345 be key to unravel the evolution of sexual conflict and its underlying mechanisms. We further
346 discuss the consequences of this novel finding for: a) our understanding of how populations
347 under strong sexual conflict respond to global warming, b) how the effects of seasonal
348 temperature fluctuations on sexual selection may contribute to maintain standing genetic
349 variation of secondary sexual traits (i.e. lek paradox), and c) how adaptation of male sexually
350 selected traits in response to thermal ecology may foster diversification and reproductive
351 barriers between populations.

352

353 First, we show that there is quick evolution of male harm to temperature after 29
354 generations of experimental evolution under different thermal regimes. We found higher
355 levels of male harm (net impact of male exposure on female fitness) at temperatures at which
356 flies had evolved to; male harm was lowest at 28°C in flies evolved in the cold regime
357 ($20 \pm 4^\circ\text{C}$) and at 20°C in those evolved in the hot regime ($28 \pm 4^\circ\text{C}$; Fig. 1). In addition, flies
358 evolved in a moderate regime ($24 \pm 4^\circ\text{C}$) exhibited similar levels of harm at 20, 24 and 28°C
359 despite the fact that flies from the ancestral wild population exhibit substantially higher levels

360 of harm at 24 than at 20 or 28°C (Londoño-Nieto et al., 2023). In short, we found evidence
361 that males across replicates evolved in parallel to be more harmful to females at their evolved
362 thermal environment, as expected under adaptation given that strong sexual selection in
363 males has led to the evolution of male harm in this species (Holland & Rice, 1999; Kawecki
364 & Ebert, 2004; Rice, 1996). This findings contribute to the growing body of evidence
365 indicating that adaptation to novel environments can affect the level of sexual conflict
366 (Martinossi-Allibert et al., 2018).

367 Second, we report strong evidence of fast divergent evolution of behavioural vs.
368 sperm-related male traits involved in male harm at cold vs. hot regimes. Male harassment of
369 females (pre-copulatory harm) evolved to be considerably less intense and thermally plastic
370 in populations adapted to the cold regime. In contrast, seminal fluid proteins (SFPs)
371 characterized the evolution of male seminal proteomes at hot vs. cold or moderate regimes
372 (Figs. 3-5). This included proteins such as Semp1 and ovulin, which can be harmful to
373 females (Wigby & Chapman, 2005). In fact, comparison of all SFPs across thermal regimes
374 revealed that several proteins from the sex peptide and ovulin networks characterize
375 evolutionary responses to the hot regime (Fig 5). This finding strongly suggests that
376 temperature is likely to be a determining factor in the diversification of traits involved in
377 male harm in *Drosophila* and, potentially, other ectotherms. The evolution of decreased male
378 harassment at cold regime could be explained, at least partly, by natural selection acting on
379 metabolic rates, with downstream sex-specific effects on sexual selection processes (Arnqvist
380 et al., 2022). Recent theoretical and empirical developments place metabolism as a causative
381 nexus in the evolutionary interplay between ecology, life history, and sexual selection
382 (Arnqvist et al., 2022; Burger et al., 2019). Metabolic rate is intimately bound to temperature
383 across the tree of life, particularly in ectotherms (Brown et al., 2004). Thus, cold
384 temperatures may place a general constraint and/or simply increase the costs of male activity,

385 consequently affecting harassment of females in ectotherms, such that both evolutionary and
386 plastic responses to cold may generally shift male-male competition towards the post-
387 copulatory arena. In accordance with this idea, the evolution of substantially lower levels of
388 harassment to females in the cold experimental evolution regime parallels the plastic
389 reduction of harassment in response to cold temperature observed in the ancestral population
390 (Londoño-Nieto et al., 2023).

391 In contrast, there is ample evidence that hot temperatures have particularly strong
392 effects on proteins and sperm phenotype and function across animals (Dougherty et al., 2024;
393 Reinhardt et al., 2015; Sales et al., 2018). For example, high temperatures lead to a reduction
394 in sperm production, motility, viability and longevity (Wang & Gunderson, 2022). Moreover,
395 high temperatures increase entropy, affecting protein folding and reducing the fraction of
396 functional proteins (Berger et al., 2021), and recent findings suggest that hot temperature may
397 also impact seminal fluid proteins (Canal Domenech & Fricke, 2022; Martinet et al., 2023).
398 This seems to suggest that hot temperatures may be particularly constraining for post-
399 copulatory sexual selection. Indeed, our results show that temperature does affect both plastic
400 and evolutionary responses of SFPs in *Drosophila*. We found that SFPs responded
401 differentially to evolution at hot regime and, in a recent study with flies from the same
402 ancestral population, we show that hot temperature (28°C) compromises SFPs effects on
403 female receptivity (Londoño-Nieto et al., 2023). This suggests that plastic SFP responses to
404 hot temperature are maladaptive in the ancestral wild population, and that SFPs of flies
405 evolved at hot regime seem to evolve quickly to maximise male fitness, potentially affecting
406 male harm to females. Incidentally, our results add to the emerging idea that the net fitness
407 consequences of SFPs to females (i.e. whether they are beneficial, neutral or costly) largely
408 depend on environmental conditions (Hopkins & Perry 2022). To conclude, our results show
409 that evolutionary responses to coarse-grained but natural temperature fluctuations can drive

410 the divergent evolution of male traits involved in male harm to females. We suggest that
411 these responses may be widespread across the tree of life, potentially explaining the diversity
412 of traits involved in male harm across taxa and fostering speciation by contributing to
413 establish reproductive barriers among populations.

414 Third, quick divergent evolution of pre- and copulatory mechanisms of harm would
415 only be possible via strong selection operating on high levels of standing genetic variation in
416 the ancestral population (Anderson, 2012). One possibility is that such high levels of standing
417 genetic variation on male secondary sexual traits are maintained in the ancestral population
418 via adaptive phenotypic plasticity (West-Eberhard, 2003). This is consistent with the recent
419 finding, in *Drosophila* from this wild population, of high levels of thermal plasticity in both
420 pre- and copulatory harm traits within the same range of temperatures studied here (Londoño-
421 Nieto et al., 2023). As stated above, male flies in the ancestral wild population respond to
422 cold temperature by decreasing harassment to females, and flies evolved under the cold
423 temperature regime evolved to harass females less and their harassment was less plastic in
424 response to temperature variation. The clear loss of ancestral plasticity is suggestive of
425 processes maintaining adaptive phenotypic plasticity in the ancestral wild population. An
426 interesting possibility is that regular/predictable temperature fluctuations at a fine-grained
427 ecological scale (e.g. circadian variation) in nature may, via temperature effects on sexual
428 selection in males, contribute to maintain high levels of thermal adaptive phenotypic
429 plasticity in secondary sexual traits. Such plasticity could, in turn, allow for substantial levels
430 of cryptic genetic variation on which later directional selection could operate (e.g. via
431 selective sweeps and/or genetic assimilation), which could explain the evolutionary responses
432 in our experimental populations. However, as discussed above plastic SFPs responses to hot
433 temperatures in the ancestral population appeared maladaptive (Londoño-Nieto et al., 2023).
434 Furthermore, we report clear evidence of strong GxE interactions in thermal reaction norms

435 for the reproductive success of male genotypes derived from our ancestral wild population,
436 estimated under strong sexual selection, that were mostly characterized by clear quadratic
437 reaction norms of opposing sign (Fig. S4). This suggests the existence of fitness trade-offs
438 and, potentially, the operation of some sort of balancing selection in the ancestral population.

439 There is piling evidence for seasonal balancing selection in *Drosophila* in traits under
440 natural selection, mostly driven by adaptation to starvation, temperature stress and the
441 seasonal boom-and-burst population dynamics typical of this and other invertebrate species
442 (Bergland et al., 2014; Hoffmann et al., 2005; Machado et al., 2021; Rudman et al., 2022).
443 Our results open the possibility of similar balancing selection via sexual selection processes,
444 which could contribute to explain the maintenance of high levels of additive genetic variation
445 on male secondary sexual traits, a classic conundrum in evolutionary biology (i.e. the “lek
446 paradox”; Kirkpatrick & Ryan, 1991). Thus, balancing selection in males may be at least
447 partly characterised by trade-offs that involve sexual selection processes, such as for example
448 investment in pre- vs. post-copulatory competition in cold vs. hot temperatures. An arising
449 prediction of this idea is that we would expect sexual differences in the type of trade-offs that
450 result from balancing selection in the wild. In accordance, temperature clines have led to a
451 negative association between resistance to starvation and cold resistance in female, but not
452 male, *Drosophila melanogaster* (Hoffmann et al., 2002,2005). We suggest future studies
453 should investigate the role that temperature effects on sexual selection may play in sex-
454 specific balancing selection, and the resulting maintenance of additive genetic variation in
455 male secondary sexual traits.

456 **Conclusions**

457 Our results show that temperature may be an important abiotic ecological factor in the
458 evolution of male harm, with implications for research on adaptation to global warming, the

459 maintenance of variability in secondary sexual traits and the diversification of male harm
460 mechanisms across populations. In addition, the finding that the male seminal proteome
461 evolves rapidly in response to temperature, and that this response is characterized by the
462 differential expression of SFPs in males evolved at the hot regime, may have implications for
463 the study of temperature effects on fertility (e.g. thermal fertility limits). We suggest future
464 research should further study plastic and evolutionary responses of SFPs to temperature, and
465 ensuing effects on female reproduction and fertility at large. Finally, here we used an
466 experimental evolution approach that largely arrests the evolution of female resistance to
467 male harm, but a priority for future research should be to understand whether and how
468 temperature may affect the evolution of female resistance to harm.

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Table 1. Sample sizes (number of vials) for female reproductive success and survivorship experiments. For behaviour assays, we observed a subset of 30 vials per replicate per each treatment combination (120 total).

Temperature treatment	Mating system	Regime of evolution														
		Cold					Moderate					Hot				Total
		Replicate				Total	Replicate				Total	Replicate				
		A	B	C	D		E	F	G	H		I	J	K	L	
20°	Monogamy	42	41	42	41	166	43	41	41	43	168	39	38	41	39	157
	Polyandry	42	43	43	43	171	43	43	38	43	167	44	43	41	39	167
24°	Monogamy	42	32	43	45	162	41	40	39	42	162	38	39	41	35	153
	Polyandry	44	38	36	45	163	42	43	39	41	165	36	35	40	41	152
28°	Monogamy	43	43	43	36	165	42	41	44	45	172	43	44	42	36	165
	Polyandry	43	41	44	35	163	44	44	40	45	173	45	44	44	36	169

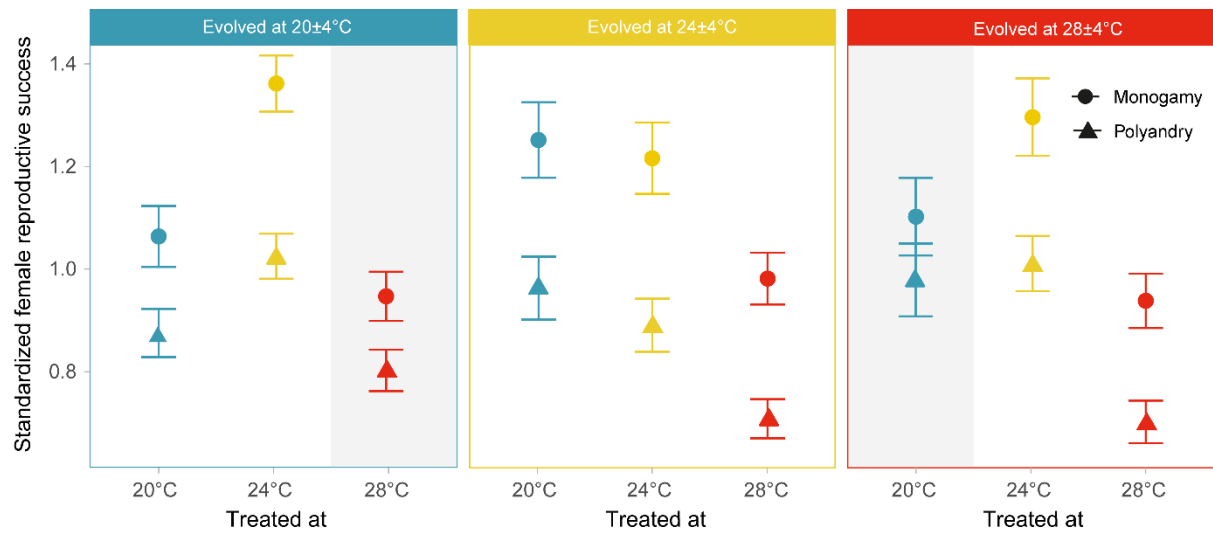


Figure 1 | Effect of mating system, temperature treatment and experimental evolution regime on female fitness. Female reproductive success (mean \pm s.e. of four replicates) across mating systems (monogamy and polyandry), temperature treatments (20, 24 and 28°C) and experimental evolution thermal regimes (20 \pm 4, 24 \pm 4 and 28 \pm 4°C). Male harm, indicated by the comparison of female reproductive success between monogamy and polyandry, was higher when flies were treated at temperatures within the thermal regime of evolution, compared to those outside this range (shaded panels). Data for each experimental evolution regime was standardized by dividing each value by the mean of the regime.

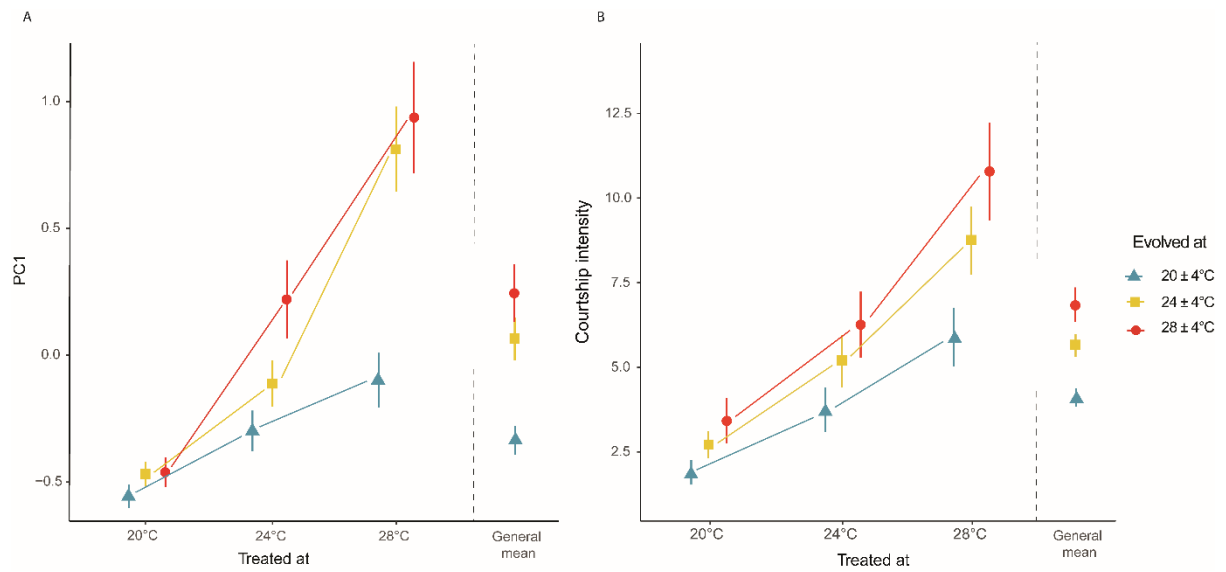


Figure 2 | Effect of temperature treatment and experimental evolution regime on pre-copulatory male harm. A) Mean (\pm s.e.) for PC1 from a PCA including behaviours causally related with pre-copulatory harm (courtship intensity, female rejection and male-male aggression), for assays at increased sexual conflict (i.e. polyandry) across experimental evolution regimes. We took this PC1 as an overall index of male-male competition and harassment to females. B) Mean (\pm s.e.) for overall courtship intensity for assays at increased sexual conflict (i.e. polyandry) across experimental evolution regimes.

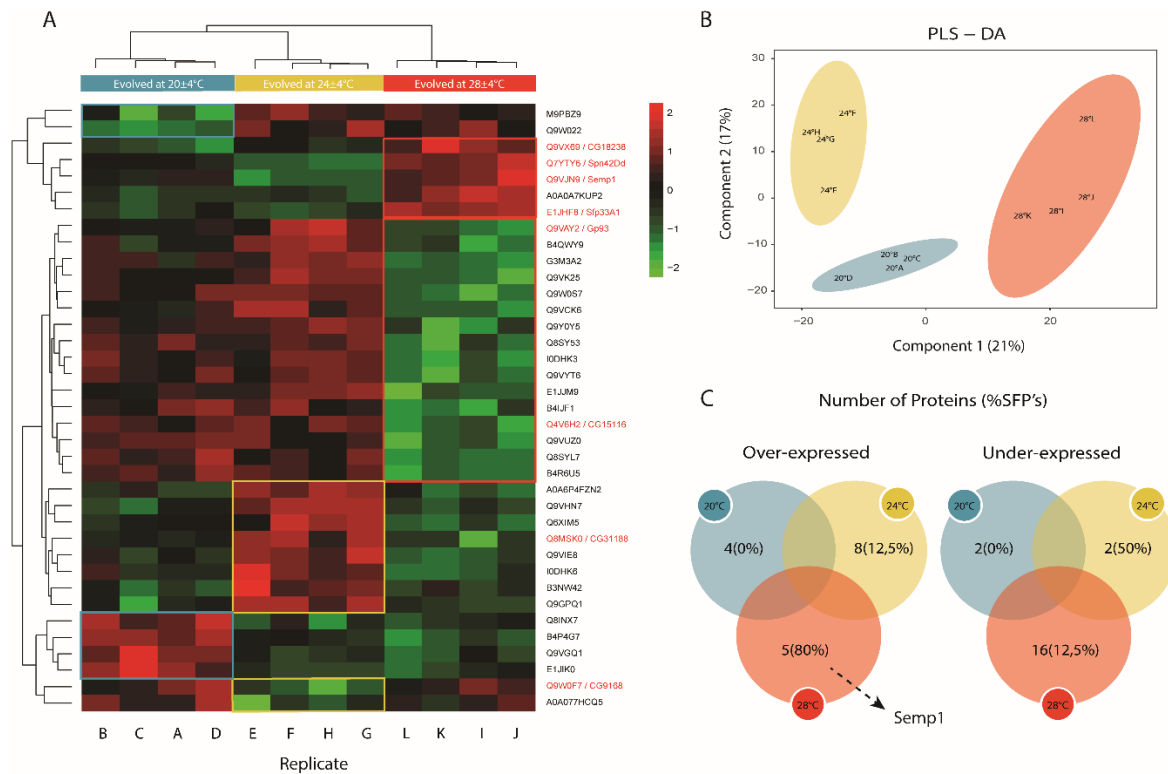


Figure 3 | Effect of experimental evolution regime on virgin males' seminal proteome. A) Heatmap showing the abundance of 37 proteins selected by the Elastic net regression. Each cell gives the across-biological replicate mean for that protein in each experimental evolution thermal regime and replicate. Boxes denote proteins singularly over and under-expressed at each experimental evolution thermal regime. B) PLS – DA plot of the proteins. Points represent all samples according to experimental evolution thermal regime and replicate. Ellipses denote variability among samples. C) Venn diagrams showing the number of proteins over and under-expressed (within the 37 proteins selected), and the corresponding percentage of SFPs, by males evolved in each experimental evolution thermal regime. Semp1 protein (Q9VJN9) was singularly over-expressed by males evolved in the hot regime. It is a seminal fluid metalloprotease which is transferred to females during mating and is required to process ovulin (Acp26Aa) and a protein which is essential for sperm storage (Acp36DE) in mated females.

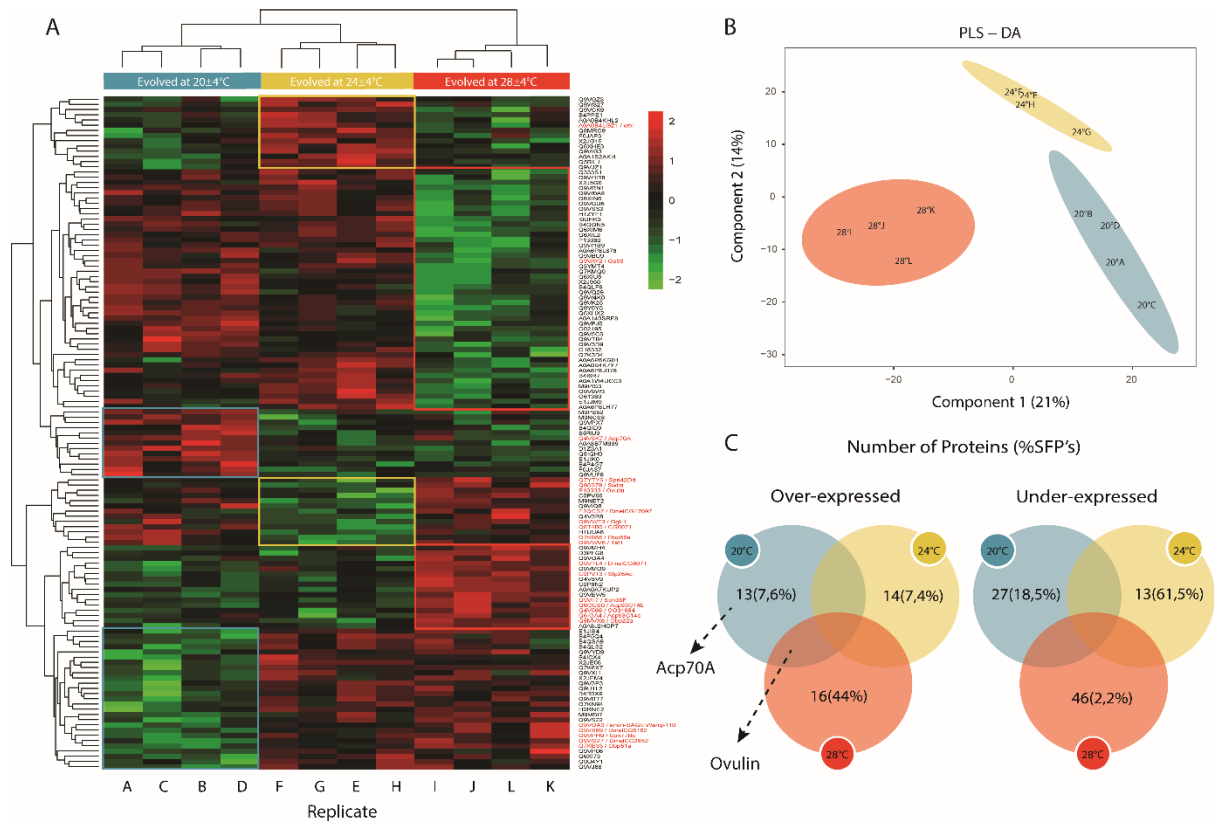


Figure 4 | Effect of experimental evolution regime on mated males' seminal proteome. A) Heatmap showing the abundance of 129 proteins selected by the Elastic net regression. Each cell gives the across-biological replicate mean for that protein in a given experimental evolution thermal regime and replicate. Boxes denote proteins singularly over and under-expressed at each experimental evolution thermal regime. B) PLS – DA plot of proteins. Points represent all samples according to experimental evolution thermal regime and replicate. Ellipses denote variability among samples. C) Venn diagrams showing the number of proteins over and under-expressed (inside the 129 proteins selected), and the corresponding percentage of SFPs, by males evolved in each experimental evolution thermal regime. Ovulin, a seminal fluid which enhances ovulation in female *Drosophila* by stimulating the release of oocytes by the ovary following mating, was over-expressed by males evolved in the hot regime. Acp70A, a seminal fluid which regulates female receptivity, was over-expressed by males evolved in cold regime.

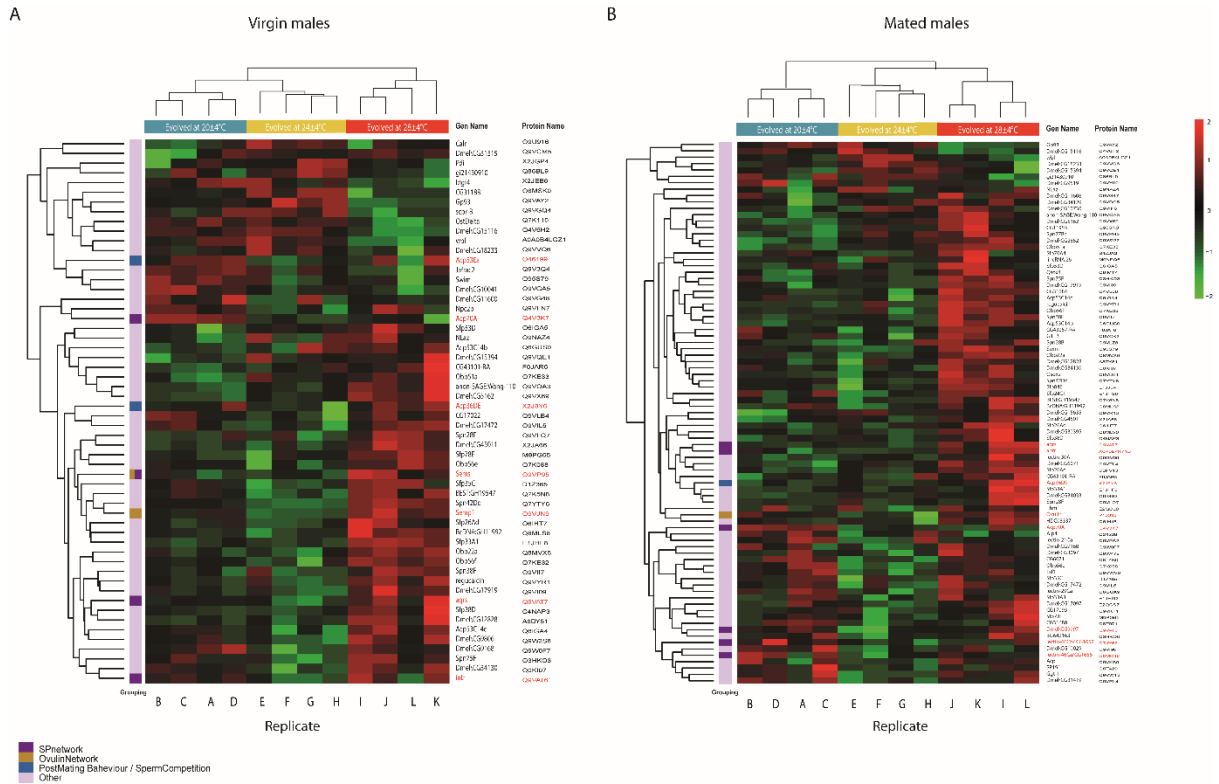


Figure 5 | Effect of experimental evolution regime on SFPs of virgin and mated males. A) Heatmap showing the abundance of 56 proteins selected by the Elastic net regression for virgin males. **B)** Heatmap showing the abundance of 85 proteins selected by the Elastic net regression for mated males. Each cell gives the across-biological replicate mean for that protein in a given experimental evolution thermal regime and replicate. Boxes denote proteins singularly over and under-expressed at each experimental evolution thermal regime. Row annotations provide functional information relating to protein functions as part of the sex peptide or ovulin networks or other known roles in sperm competition.

1 **Supplementary Information**

2 **Materials and Methods**

3 Experimental evolution design

4 Experimental evolution started in February 2020 for all populations, and finished in August
5 2021 for the hot regime, in October 2021 for the moderate regime and in April 2022 for the
6 cold regime. Specifically, four populations (replicates A, B, C and D) evolved at $20\pm 4^{\circ}\text{C}$
7 (cold regime fluctuating daily between 16 and 24°C); four populations (replicates E, F, G
8 and H) at $24\pm 4^{\circ}\text{C}$ (moderate regime fluctuating daily between 20 and 28°C), and four
9 populations (replicates I, J, K and L) at $28\pm 4^{\circ}\text{C}$ (hot regime fluctuating daily between 24
10 and 32°C) (Fig. S1). Sample size was $N = 200$ (100 males and 100 females) for all
11 populations. Providing specific estimates of N_e is difficult due to male and female
12 promiscuity and male-male competition (patterns of sperm competition), which would
13 affect effective population size (N_e) depending on factors such as the number of mates per
14 female and the extent of sperm displacement. For example, while male-male competition
15 can increase N_e by creating more mating opportunities, it can also lead to increased
16 variance in reproductive success. This variance may reduce N_e if highly competitive males
17 dominate fertilizations across multiple females, especially given *Drosophila*
18 *melanogaster*'s tendency for sperm displacement (strong last-male sperm precedence).
19 However, even with these dynamics, previous studies on this species indicate that a
20 population size of 100 males and 100 females (with females mating with multiple males)
21 yields $N_e \sim 150$ or higher and sufficiently large to mitigate genetic drift and inbreeding
22 (Reuter et al., 2008; Rice & Holland, 2005; Snook et al., 2009).

23 Each group was maintained in a dedicated pre-programmed incubator that controlled for its
24 specific temperature regime throughout the experimental period. All incubators were
25 identical in brand and model (Memmert IPP110plus), programmed with the same protocol,
26 and housed in the same room with consistent external temperatures. Lighting conditions
27 were exactly the same (12:12 photoperiod and same luminance profile) across incubators.
28 Each incubator had integrated sensors to monitor temperature and humidity continuously,
29 which we supplemented with additional redundant sensors (Sense Anywhere Temp+RH
30 Module model 01-01-20) to ensure precise monitoring of environmental conditions. These
31 sensors recorded data in real-time, which we monitored online daily to verify that the
32 thermal regimens, treatments, and temperature fluctuations remained stable. Finally,
33 experimental regimes were rotated across different incubators during the experimental
34 evolution period. Different developmental times at each thermal regime led to differences
35 in the duration of experimental evolution across treatments. Populations from the hot
36 regime were assayed between September and October 2021, from the moderate regime
37 between November and December 2021 and from the cold regime between June and July
38 2022. This prevented post-evolution assays from being conducted simultaneously across
39 experimental evolution regimes, but there are three main reasons why we don't expect
40 biases in our estimates across regimes. First and foremost, behavioural and fitness assays
41 incorporate internal controls as we are always comparing the drop in behaviours/fitness
42 between monogamy and polyandry, across the three temperature treatments. For our aims,
43 it is these relative changes that matter (as well as their interactions), not overall changes in
44 behavioural rates and/or fitness across experimental evolution regimes. This also explains
45 why we standardized our data within each experimental evolution regime and why we
46 never attempted to compare overall levels of harm or associated behaviours across

47 experimental evolution regimes. We standardized the data within each evolutionary thermal
48 regime by dividing each observation by the overall mean for that regime. By standardizing
49 within each regime, we ensure that we compare relative changes within experimental
50 evolution treatments. This approach emphasizes variability and patterns within each regime
51 relative to its own baseline, facilitating clearer comparisons across regimes in terms of
52 relative (e.g. how much fitness drops between monogamy and polygamy and/or across the
53 three temperature treatments, and the interaction between these two) and not absolute
54 effects. Importantly, this also controls for any potential residual block effects (i.e.
55 independent of mating system and treated temperature) on overall fitness or behavioural
56 rates across experimental regimes, which is not the focus of our analysis. Second, we were
57 meticulous about standardizing lab conditions to ensure a common garden. All post-
58 evolution assays followed identical protocols. The flies used in each assay were of the same
59 age, and temperature treatments were managed under strictly controlled conditions. As
60 previously explained, we used the same incubators (Memmert IPP110plus), equipped with
61 both integrated and supplementary sensors for redundant monitoring of temperature and
62 humidity in real-time, allowing for daily online verification to ensure temperature stability
63 throughout the treatments. Female offspring were similarly incubated under identical
64 temperature controls before being frozen for subsequent counting. Lighting conditions were
65 exactly the same (12:12 photoperiod and luminance profile). All behavioural and proteomic
66 assays were conducted in the same temperature controlled (TC) room, with the same
67 lighting, and constant temperature and humidity control with redundant sensors and a
68 portable high-sensitive probe to ensure consistent environmental conditions in the panels
69 where vials were assayed, within the TC room. We also ensured that all assays were
70 conducted in days with good weather, to control for the potential influence of drops in

71 barometric pressure on mating behaviour (see Table S1 below). Third, for proteomic
72 quantification, all assayed males across experimental evolution regimes were dissected at
73 the same time (i.e. in balanced order across the same days) and proteomic analyses
74 conducted at the same time for all samples, to avoid block effects.

75 Behavioural assays (experiment 1)

76 Immediately after the fitness experiment started, we conducted behavioural observations on
77 the first day of the experiment across all temperature treatments. We conducted behavioural
78 observations in the same TC room, so we had to conduct trials at 20°C, 24°C, and 28°C
79 over three consecutive days (with both monogamy and polyandry treatments and the 4
80 replicates evaluated at the same time for each temperature -240 vials-). Note that we
81 collected virgin flies over three consecutive days to ensure all flies were 4-5 days-old at the
82 start of the experiment. Observations started at lights-on (9 a.m.) and lasted for 8 hr, during
83 which time we continuously recorded reproductive behaviours using scan sampling of vials
84 with an all-occurrences recording rule. Scans consisted of observing all vials in succession
85 for approximately 3 seconds each (i.e. one scan per vial every ~ 12 minutes), during which
86 we recorded all instances of the behaviours specified in the main text. Observers were blind
87 to the population replicate but not to the sociosexual context (i.e. monogamy vs. polygamy,
88 obvious from observing the vial) or the experimental evolution regime (i.e. due to trials
89 being conducted at different times; see above).

90 Male harm and behavioural analysis (experiment 1)

91 In all cases, we assessed fit and validated models by visual inspections of diagnostic plots
92 on raw and residual data (Zuur et al., 2010). For reproductive success, we used normal

93 distribution with an “*identity*” as link function. For courtship and aggression rates, a zero
94 inflated distribution was applied, while for female rejection rate, we used a negative
95 binomial distribution with “*log*” as link function. Graphical inspection of the modelled
96 component 1 of the PCA, revealed that the normality assumption was apparently violated.
97 Natural logarithm transformation solved these problems and allowed us to run a LMM with
98 a Gaussian error distribution and an “*identity*” as link function. As our replicates are from
99 different populations, we also fitted random slopes models for correlated fixed effects of
100 temperature evolution regime and temperature treatment (Arnqvist, 2020). However, in all
101 cases we found that fixed slopes models presented the minimum AICc value, supporting
102 them as the best models given the trade-off between fit to the data and model complexity
103 (Konishi & Kitagawa, 2008); but we note results did not change qualitatively in either case.
104 We performed model selection by backward stepwise elimination; refitting models without
105 the triple interaction where necessary to arrive at the minimal adequate model. Replicate
106 population was kept on all analyses to control for this variation. We also run post hoc
107 Tukey’s test as an additional way to explore interactions while controlling for inflation of
108 experiment-wise type 1 error rate.

109 Proteomics assays (experiment 2)

110 *Proteomics sample preparation*

111 Protein extraction and preparation of the SWATH experiment (library and samples) were
112 carried out in the proteomics laboratory of the University of Valencia, Spain, according with
113 the procedure indicated below.

114 Total protein extracts were prepared by centrifugation of each sample at 13000 rpm 15 min.
115 Supernatants were discarded and pellets suspended in 50 μ L of Laemmli buffer 1.5 X. Vortex
116 5 min and sonicated 5 min. Total protein concentration was calculated using Machery Nagel
117 kit. To prepare library and each sample for SWATH experiment appropriate volume of
118 sample (7.5 μ g/sample to SWATH and 25 μ g of mixed samples to perform library) was
119 denatured at 95°C during 5 min.

120 *Spectral Library Building*

121 Aliquots with an equivalent amount of a selection of samples were mixed to make a pool for
122 building the spectral library (25 μ g). The library electrophoresis was performed using a 12%
123 precast gel (Bio-Rad) at 200V for 30 min. Gels were fixed with 40% ethanol/10% acetic acid
124 for one hour and stained with colloidal Coomassie (Bio-Rad) for 15 min. Gels were destained
125 with H₂O milliQ and cutted into six pieces for protein digestion.

126 In gel protein digestion

127 The career corresponding to the library was cutted into 6 pieces and then was digested with
128 sequencing grade trypsin (Promega) as described by Shevchenko et al., 1996. 500 ng of
129 trypsin were used for each sample, and digestion was set to 37°C on. Trypsin digestion was
130 stopped with 10% TFA, the SN was removed, and the library gel slides were dehydrated with
131 pure ACN(Shevchenko et al., 1996). The new peptide solutions were combined with the
132 corresponding SN. The peptide mixtures were dried in a speed vacuum and re suspended in
133 2% ACN; 0.1% TFA (15 μ L) before LC-MS/MS (Liquid chromatography and tandem mass
134 spectrometry/mass spectrometry) analysis.

135 *LC-MS/MS analysis*

136 Peptides were analysed using an Ekspert nanoLC 425 nanoflow system (Eksigent
137 Technologies, ABSCIEX) coupled to a mass spectrometer nanoESI qTOF MS (6600 plus
138 TripleTOF, ABSCIEX). 5 µl of peptide mixture sample was loaded onto a trap column (3µ
139 C18-CL, 350 µm x 0.5 mm; Eksigent) and desalted with 0.1% TFA at 5 µl/min during 5 min.
140 Peptides were then loaded onto an analytical column (3 µ C18-CL 120 Å, 0.075 x 150 mm;
141 Eksigent) equilibrated in 5% acetonitrile 0.1% FA (formic acid). Elution was carried out with
142 a linear gradient of 7 to 40% B in A for 120 min. (A: 0.1% FA; B: ACN, 0.1% FA) at a flow
143 rate of 300 nL/min. Samples were ionized in a Source Type: Optiflow < 1 µl Nano applying
144 3.0 kV to the spray emitter at 200 °C. Analysis was carried out in a data-dependent mode.
145 Survey MS1 scans were acquired from 350–1400 m/z for 250 ms. The quadrupole resolution
146 was set to ‘LOW’ for MS2 experiments, which were acquired 100–1500 m/z for 25 ms in
147 ‘high sensitivity’ mode. Following switch criteria were used: charge: 2+ to 4+; minimum
148 intensity; 250 counts per second (cps). Up to 100 ions were selected for fragmentation after
149 each survey scan. Dynamic exclusion was set to 15 s. The rolling collision energies equations
150 were set for all ions as for 2+ ions according to the following equations:
151 $|CE|=(\text{slope})x(m/z)+(\text{intercept})$. The system sensitivity was controlled by analysing 500 ng
152 of K562 trypsin digestion (Sciex). The system sensitivity was controlled with 2 fmol
153 PepCalMix (LC Packings).

154 *Protein Identification*

155 ProteinPilot default parameters were used to generate peak list directly from 6600 TripleTof
156 wiff files. The Paragon algorithm (Shilov et al., 2007) of ProteinPilot v 5.0 search engine
157 (ABSciex) was used to search the Uniprot_insecta and Uniprot_Drosophila database with the

158 following parameters: Trypsin specificity, IAM cys-alkylation and the search effort set to
159 through and FDR correction.

160 The protein grouping was done by Pro group algorithm: A protein group in a Pro Group
161 Report is a set of proteins that share some physical evidence. Unlike sequence alignment
162 analyses where full-length theoretical sequences are compared, the formation of protein
163 groups in Pro Group is guided entirely by observed peptides only. Since the observed
164 peptides are determined from experimentally acquired spectra, the grouping can be
165 considered to be guided by usage of spectra. Then, unobserved regions of protein sequence
166 play no role in explaining the data.

167 *SWATH analysis of individual samples*

168 For individual SWATH analysis 7.5 µg of total protein extract was loaded in a
169 1D_SDS_PAGE gel to clean and concentrate samples. Gel fraction was cut and the sample
170 was digested with sequencing grade trypsin (Promega) as described elsewhere (Shevchenko
171 et al., 1996). 500 ng of trypsin in 100 µl of ABC solution was used. The digestion was stopped
172 with TFA (1% final concentration), a double extraction with ACN was done and all the
173 peptide solutions and dried in a rotatory evaporator. Sample was re suspended with 15 µL of
174 2% ACN; 0.1% TFA.

175 *SWATH LC-MS/MS Analysis*

176 5 µl of each sample were loaded onto a trap column (3µ C18-CL 120 Å, 350 µm x 0.5mm;
177 Eksigent) and desalted with 0.1% TFA at 5 µl/min during 5 min. Peptides were loaded onto
178 an analytical column (3µ C18-CL 120 Å, 0.075 x 150 mm; Eksigent) equilibrated in 5%
179 acetonitrile 0.1% FA (formic acid). Peptide elution was carried out with a linear gradient of

180 7 to 40% B in 120 min (A: 0.1% FA; B: ACN, 0.1% FA) for at a flow rate of 300 nl/min.

181 Peptides were analysed in a mass spectrometer nanoESI qTOF (6600plus TripleTOF,
182 ABSCIEX).

183 Sample was ionized in a Source Type: Optiflow < 1 µl Nano applying 3.0 kV to the spray
184 emitter at 200°C. The tripleTOF was operated in swath mode, in which 0.050-s TOF MS scan
185 from 350–1250 m/z was performed, followed by 0.080-s product ion scans from 350–1250
186 m/z. 100 variable windows from 400 to 1250 m/z were acquired throughout the experiment.
187 The total cycle time was 2.79 secs. The individual SWATH injections were randomized.

188 *Protein quantification*

189 The wiff files obtained from SWATH experiment were analysed by Peak View 2.2. The
190 processing settings used for the peptide selection were: 20 peptides per protein, 6 transitions
191 per peptide, 95% peptide confidence threshold, 1.0% false discovery rate threshold, peptides
192 modified excluded, 5 min XIC extraction window and 25 ppm XIC width.

193 Retention times of the detected peptides were alienate using major proteins to calibrate
194 retention times. With the extraction parameters of the areas used, proteins (FDR <1%) were
195 quantified in the 72 samples.

196 *Data analysis*

197 We normalized the protein areas calculated by the total sum of the areas of all the quantified
198 proteins. We used an elastic net penalized logistic regression model to analyse our data sets.
199 The elastic net regression is a hybrid method that combines features of Lasso and Ridge
200 regularization techniques. This method is particularly suited for situations where the number
201 of predictors far exceeds the number of observations, as it performs both regularization and

202 variable selection. The elastic net is controlled by two key parameters: α , which balances the
203 contribution of Lasso ($\alpha=1$) and Ridge ($\alpha=0$) regularization, and λ , which determines the
204 overall strength of the penalization. The optimal value of α and λ is typically estimated using
205 cross-validation (Zou & Hastie, 2005). The optimal values for our analyses are in the Table
206 S10

207 GxE assay (experiment 3)

208 We used Nakagawa's R-squared (Nakagawa & Schielzeth, 2013) to extract the variance
209 explained by each model, analysed random effects using *raneef* function from lme4 (Bates et
210 al., 2015), and tested via likelihood ratio tests for significance. For reproductive success of
211 focal males we used beta distribution with “*logit*” as link function.

212

213 **Supplementary references**

214

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250

251 **Results**

252 Behavioral assays (experiment 1)

253 Analysing behaviours involved in male harassment separately, as well as effects across
254 mating system, yielded qualitatively very similar results to those reported in the main
255 manuscript (using PC1 of a PCA on all behaviours known to play a role in pre-copulatory
256 harm). First, experimental evolution regime affected overall aggression ($X^2_2 = 10.18$, $P =$
257 0.006) and female rejection rate ($X^2_2 = 9.31$, $P = 0.009$), with lower levels of both variables
258 at colder regimes (Figs. S5-8). Courtship rate and rejection rate per courtship exhibited a
259 trend in the same direction, but the effect was not significant (Figs. S9-12), suggesting that
260 the increase in male avoidance behaviour could imply an increase in female probability to
261 reject male courtships. However, the interpretation of the female rejections per courtship
262 requires caution, given that its calculation was only possible 30% of the observation time
263 (when courtship rate differs from 0). Second, we found that flies evolved at the cold
264 thermal regime were less thermally plastic for aggression than flies from the other regimes
265 (i.e., flatter reaction norms: experimental evolution x temperature treatment interaction: X^2_2
266 $= 11.81$, $P = 0.018$; Figs. S4-5 and Table S6). Courtship intensity exhibited a clear trend in
267 the same direction as aggression rate (Figs. S11-12) albeit this effect was not significant
268 (experimental evolution x treatment temperature interaction: $X^2_4 = 6.21$, $P = 0.183$). Female
269 rejection (experimental evolution x temperature treatment interaction: $X^2_4 = 12.52$, $P =$
270 0.013) and female rejection per courtship (experimental evolution x temperature treatment
271 interaction: $X^2_4 = 11.76$, $P = 0.019$) exhibited less thermal plasticity in flies evolved at
272 moderate regime (Figs. S7-10 and Table S7). Finally, courtship rate varied greatly across
273 mating systems and the strength of this effect varied considerably across temperature

274 treatments (mating system x treatment temperature interaction: $X^2_2 = 18.37$, $P < 0.001$),
275 suggesting less harassment in flies treated at 20°C and more in flies treated at 28°C (Figs.
276 S11-12; Table S8).

277 Proteomics assays (experiment 2)

278 The parameters used for the analysis of the whole proteome and the subset of seminal fluid
279 proteins for both virgin and mated males are specified in the Table S10.

280

281 **Table S1.** Range of atmospheric pressure (hPa) recorded during experimental periods for each
 282 experimental evolution regime. Behavioural observations and proteomics experiments were
 283 conducted over a shorter duration of 3 days, while the male harm experiment lasted six weeks.

284

	28±4°C		24±4°C		20±4°C	
	Min	Max	Min	Max	Min	Max
Behavioural observations	1011,6	1018,7	1007	1016,7	1004,4	1012,5
Proteomics	1008,2	1020,5	1000,9	1017,5	1001,3	1007,6
Male Harm Experiment	996,7	1020,5	994,2	1023,9	1002,9	1016,1
Mean ± SD						
Male Harm Experiment	1013,16 ± 4,01	1008,88 ± 4,70	1013,93 ± 6,49	1008,72 ± 7,30	1011,13 ± 2,12	1007,83 ± 2,32

285

286 **Table S2.** Summary statistics from a PCA conducted on reproductive behaviours for polyandry
 287 mating system. a) variance, eigenvalue, and loadings associated with the three principal components
 288 (PCs). b) summary statistics from fitting linear mixed models for each experimental evolution regime
 289 due to its significant interaction with temperature treatment c) temperature treatment contrast table
 290 from Tukey's post hoc from the full model as an additional way to explore the interaction.
 291

292 a)

		<i>PC1</i>	<i>PC2</i>	<i>PC3</i>
Variance explained (%)		67.72	22.86	9.42
Eigenvalue		2.03	0.68	0.28
Loadings	Courtship rate	0.63	0.28	0.73
	Aggression rate	0.48	-0.87	
	Rejection rate	0.61	0.40	-0.68

293

294 b)

Experimental evolution regime	<i>PCI</i>				
	<i>Effect</i>	<i>F</i>	<i>Df</i>	<i>Df.res</i>	<i>P value</i>
20±4°C	Temperature treatment	11.71	2	347	< 0.001
24±4°C	Temperature treatment	32.88	2	345	< 0.001
28±4°C	Temperature treatment	27.21	2	347	< 0.001

295

296 c)

Experimental evolution regime	<i>PCI</i>					
	<i>Contrast</i>	<i>Estimate</i>	<i>SE</i>	<i>df</i>	<i>t-value</i>	<i>P value</i>
20±4°C	20° – 24°	-0.31	0.117	1039	-2.64	0.022
	28° – 24°	0.19	0.117	1039	1.62	0.237
	28° – 20°	0.50	0.116	1039	4.30	< 0.001
24±4°C	20° – 24°	-0.32	0.117	1039	-2.74	0.017
	28° – 24°	0.62	0.117	1039	5.29	< 0.001
	28° – 20°	0.94	0.117	1039	8.03	< 0.001
28±4°C	20° – 24°	-0.47	0.117	1039	-4.00	< 0.001
	28° – 24°	0.47	0.116	1039	4.09	< 0.001
	28° – 20°	0.94	0.117	1039	8.03	< 0.001

297

298 **Table S3.** a) Summary statistics from fitting linear mixed models for each experimental evolution
 299 regime due to significant interactions between experimental evolution regime and both temperature
 300 treatment and mating system. b) polyandry – monogamy and c) temperature treatment contrast table
 301 from Tukey’s post hoc from the full model as an additional way to explore interactions. d) statistical
 302 test of non-significant results.

303 a)

Experimental evolution regime	<i>Female reproductive success</i>				
	<i>Effect</i>	<i>F</i>	<i>Df</i>	<i>Df₂</i>	<i>P value</i>
20±4°C	Temperature treatment	28.37	2	983.24	<0.001
	Mating system	33.08	1	983.03	<0.001
24±4°C*	Temperature treatment	0.96	2	1.99	0.510
	Mating system	40.51	1	994.59	<0.001
28±4°C	Temperature treatment	14.60	2	956.11	<0.001
	Mating system	18.56	1	956.01	<0.001

304 *For this thermal regime random slopes model presented the minimum AICc value

305 b)

Experimental evolution regime	<i>Female reproductive success</i>				
	<i>Estimate</i>	<i>SE</i>	<i>Df₂</i>	<i>t-value</i>	<i>P value</i>
20±4°C	21.4	5.45	2937	3.930	<0.001
24±4°C	39.7	5.40	2937	7.339	<0.001
28±4°C	26.2	5.53	2937	4.733	<0.001

306

307 c)

308

Experimental evolution regime	<i>Female reproductive success</i>					
	<i>Contrast</i>	<i>Estimate</i>	<i>SE</i>	<i>Df₂</i>	<i>t-value</i>	<i>P value</i>
20±4°C	20° – 24°	-21.72	6.67	2940	-3.265	0.003
	20° – 28°	12.32	6.65	2939	1.852	0.153
	24° – 28°	34.04	6.72	2941	5.064	<0.001
24±4°C	20° – 24°	7.15	6.66	2939	1.073	0.530
	20° – 28°	34.61	6.58	2939	5.264	<0.001
	24° – 28°	27.46	6.62	2939	4.150	<0.001
28±4°C	20° – 24°	-13.84	6.84	2939	-2.023	0.106
	20° – 28°	25.47	6.69	2939	3.808	<0.001
	24° – 28°	39.31	6.79	2940	5.785	<0.001

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312

d)

<i>Female reproductive success</i>				
<i>Effect</i>	<i>F</i>	<i>Df</i>	<i>Df₂</i>	<i>P value</i>

Experimental evolution regime x temperature treatment x mating system	0.423	4	2933	0.791
Temperature treatment x mating system	1.792	2	2933	0.166

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315 **Table S4.** a) Summary statistics from fitting Cox PH mixed models separately for each experimental
 316 evolution regime due to significant interactions between experimental evolution regime and both
 317 temperature treatment and mating system. b) contrast table from Tukey's post hoc from the full model
 318 as an additional way to explore experimental evolution regime x temperature treatment and mating
 319 system interaction. c) Summary statistics from fitting Cox PH mixed models separately for each
 320 temperature treatment due to significant interaction with mating system d) contrast table from
 321 Tukey's post hoc from the full model as an additional way to explore mating system x temperature
 322 treatment interaction. e) statistical test of non-significant results.

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 324
 325

a)

Experimental evolution regime	<i>Lifespan</i>			
	<i>Effect</i>	<i>Chisq</i>	<i>Df</i>	<i>P value</i>
20±4°C	Temperature treatment	134.37	2	<0.001
	Mating system	299.81	1	<0.001
24±4°C	Temperature treatment	234.34	2	<0.001
	Mating system	311.66	1	<0.001
28±4°C	Temperature treatment	310.42	2	<0.001
	Mating system	266.45	1	<0.001

326
 327

b)

Experimental evolution regime	<i>Lifespan</i>					
	<i>Contrast</i>	<i>Estimate</i>	<i>SE</i>	<i>Df₂</i>	<i>t-value</i>	<i>P value</i>
20±4°C	20° – 24°	-0.48	0.08	Inf	-5.96	<0.001
	20° – 28°	-0.87	0.08	Inf	-10.67	<0.001
	24° – 28°	-0.38	0.08	Inf	-4.86	<0.001
	Polyandry - Monogamy	-1.11	0.07	Inf	-16.430	<0.001
24±4°C*	20° – 24°	-0.80	0.09	Inf	-9.31	<0.001
	20° – 28°	-1.52	0.09	Inf	-17.59	<0.001
	24° – 28°	-0.72	0.08	Inf	-9.04	<0.001
	Polyandry - Monogamy	-1.37	0.07	Inf	-19.863	<0.001
28±4°C	20° – 24°	-0.89	0.08	Inf	-10.35	<0.001
	20° – 28°	-1.67	0.08	Inf	-19.48	<0.001
	24° – 28°	-0.78	0.08	Inf	-9.68	<0.001
	Polyandry - Monogamy	-1.27	0.07	Inf	-18.268	<0.001

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c)

Temperature treatment	<i>Lifespan</i>			
	<i>Effect</i>	<i>Chisq</i>	<i>Df</i>	<i>P value</i>
20°C	Mating system	235.28	1	<0.001
24°C	Mating system	369.63	1	<0.001
28°C	Mating system	298.31	1	<0.001

331 d)

<i>Lifespan</i>						
<i>Temperature treatment</i>	<i>Contrast</i>	<i>Estimate</i>	<i>SE</i>	<i>Df₂</i>	<i>t-value</i>	<i>P value</i>
20°C	Polyandry - Monogamy	-1.22	0.07	Inf	-16.984	<0.001
24°C		-1.40	0.07	Inf	-20.054	<0.001
28°C		-1.12	0.06	Inf	-17.225	<0.001

332

333

334 e)

335

<i>Lifespan</i>			
<i>Effect</i>	<i>Chisq</i>	<i>Df</i>	<i>P value</i>
Experimental evolution regime x temperature treatment x mating system	8.006	4	0.091

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340 **Table S5.** Seminal fluid proteins that were over (+) and under (-) expressed by experimentally
 341 evolved virgin (V) and mated (M) males in different thermal regimens with their respective molecular
 342 function and biological process.

	20 ± 4°C		24 ± 4°C		28 ± 4°C		Molecular function	Biological process
	V	M	V	M	V	M		
A0A0B4LGZ1				+				Probable chaperone protein involved in dorsoventral axis patterning in early embryos. Probably acts by folding and targeting pipe into the Golgi.
C0PV13						+		Sexual reproduction
E1JHF8					+			Sexual reproduction
E2QCS7				-			Serine hydrolase activity, triglyceride lipase	Lipid metabolic process, sexual reproduction.
P10333				-			Identical protein binding	Mating, positive regulation of octopamine signalling pathway, positive regulation of ovulation, sexual reproduction, sperm competition
Q4V3K7		+					Hormone activity	Regulation of female receptivity, post-mating behaviour
Q4V566						+	Lipase activity	Lipid catabolic process
Q4V6H2					-		Peroxidase activity, thioredoxin-dependent peroxiredoxin activity	Response to oxidative stress, sexual reproduction
Q6IGA4						+		Sexual reproduction.
Q6GUS0						+		Sexual reproduction
Q7K088				-			Odorant binding	Sensory perception of smell, sensory perception of chemical stimulus, sexual reproduction
Q7KE33		-					Odorant binding	Sensory perception of smell, sensory perception of chemical stimulus, sexual reproduction
Q7YTY6				-	+			Serine protease inhibitor with activity toward trypsin. Involved in innate immunity to fungal infection by negatively regulating the Toll signalling pathway and suppressing the expression of the antifungal peptide drosomycin. Acts upstream of SPE and grass, and downstream of the fungal cell wall pattern recognition receptor GNB3. May function specifically in the GNB3-dependent beta-1,3-glucan branch of the Toll pathway.
Q8MSK0			+				Iron ion binding, L-ascorbic acid binding, rocollagen-proline 4-dioxygenase activity	Peptidyl-proline hydroxylation to 4-hydroxy-L-proline, sexual reproduction
Q8MVX6						+	Odorant binding	Sensory perception of chemical stimulus, sexual reproduction
Q8T4B0				-			Metalloaminopeptidase activity, peptide	Peptide catabolic process, proteolysis, sexual reproduction

							binding, zinc ion binding	
Q95S79				-			Wnt-preotein binding	Positive regulation of canonical Wnt signaling pathway, positive regulation of Wnt signaling pathway by establishment of Wnt protein localization to extracellular region, proteolysis
Q9VAY2					-	-	ATP hydrolysis activity, ATP binding, ATP-dependent protein folding chaperone, unfolded protein binding	Cellular response to heat, endodermal digestive tract morphogenesis, midgut development, protein folding, ubiquitin-dependent ERAD pathway
Q9VII7						+	Serine-type endopeptidase inhibitor activity	Defence response to Gram-negative bacterium, negative regulation of peptidase activity, negative regulation of proteolysis, sexual reproduction
Q9VJN9						+		Seminal fluid metalloprotease which is transferred to females during mating and is required for processing of two other seminal fluid proteins Acp26Aa and Acp36DE in mated females.
Q9VPH9		-						Negative regulation of peptidase activity, sexual reproduction.
Q9VQA3		-						Proteolysis, sexual reproduction
Q9VTL4						+	Metalloaminopeptidase activity, peptide binding, zinc ion binding	Peptide catabolic process, proteolysis, sexual reproduction.
Q9VWT3				-			Glutathione hydrolase activity, peptidyltransferase activity	Glutathione catabolic process, glutathione metabolic process, response to light stimulus, sexual reproduction.
Q9VWV6				-			Iron ion binding	Iron ion transmembrane transport, iron ion transport, olfactory behaviour, response to fungus
Q9VX69		-				+	lipase activity, methyl indole-3-acetate esterase activity, serine hydrolase activity, triglyceride lipase activity	Lipid catabolic process, sexual reproduction
Q9W0F7			-					Sexual reproduction
Q9W227		-						PPIases accelerate the folding of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides.

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348 **Table S6.** a) Summary statistics from fitting generalized linear mixed models for each experimental
 349 evolution regime due to significant interaction between experimental evolution regime and
 350 temperature treatment for male-male aggression rate. b) temperature treatment contrast table from
 351 Tukey's post hoc from the full model as an additional way to explore the interaction.

352
 353

a)

Experimental evolution regime	<i>Aggression rate</i>			
	<i>Effect</i>	<i>Chisq</i>	<i>Df</i>	<i>P value</i>
20±4°C	Temperature treatment	0.52	2	0.770
24±4°C	Temperature treatment	24.60	2	<0.001
28±4°C	Temperature treatment	9.89	2	0.007

354
 355

b)

Experimental evolution regime	<i>Aggression rate</i>					
	<i>Contrast</i>	<i>Estimate</i>	<i>SE</i>	<i>Df₂</i>	<i>t-value</i>	<i>P value</i>
20±4°C	20° – 24°	0.16	0.33	1037	0.50	0.869
	28° – 24°	0.09	0.26	1037	0.37	0.924
	28° – 20°	-0.07	0.30	1037	-0.21	0.974
24±4°C	20° – 24°	-0.78	0.26	1037	-2.95	<0.001
	28° – 24°	0.36	0.17	1037	2.09	0.091
	28° – 20°	1.14	0.23	1037	4.88	0.009
28±4°C	20° – 24°	-0.83	0.29	1037	-2.87	0.011
	28° – 24°	0.07	0.16	1037	0.49	0.878
	28° – 20°	0.91	0.28	1037	3.30	0.002

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 357

358 **Table S7.** a) Summary statistics from fitting generalized linear mixed models for each experimental
 359 evolution regime due to significant interaction between experimental evolution regime and
 360 temperature treatment for rejection rate. b) temperature treatment contrast table from Tukey's post
 361 hoc from the full model as an additional way to explore the interaction for rejection rate. c) summary
 362 statistics from fitting generalized linear mixed models for each experimental evolution regime due to
 363 significant interaction between experimental evolution regime and temperature treatment for rejection
 364 rate per courtship. d) temperature treatment contrast table from Tukey's post hoc from the full model
 365 as an additional way to explore the interaction for rejection rate per courtship. e) statistical test of
 366 non-significant results for rejection rate.

367

368 a)

Experimental evolution regime	<i>Rejection rate</i>			
	<i>Effect</i>	<i>Chisq</i>	<i>Df</i>	<i>P value</i>
20±4°C	Temperature treatment	10.88	2	0.004
24±4°C	Temperature treatment	11.5	2	0.003
28±4°C	Temperature treatment	43.73	2	<0.001

369

370 b)

Experimental evolution regime	<i>Rejection rate</i>					
	<i>Contrast</i>	<i>Estimate</i>	<i>SE</i>	<i>Df₂</i>	<i>t-value</i>	<i>P value</i>
20±4°C	20° – 24°	-1.47	0.42	2116	-3.54	0.001
	28° – 24°	0.50	0.38	2116	1.34	0.374
	28° – 20°	1.98	0.42	2116	4.77	<0.001
24±4°C	20° – 24°	-0.39	0.39	2116	-1.00	0.573
	28° – 24°	0.31	0.37	2116	0.83	0.680
	28° – 20°	0.70	0.39	2116	1.82	0.164
28±4°C	20° – 24°	-2.15	0.40	2116	-5.32	<0.001
	28° – 24°	0.18	0.36	2116	0.52	0.862
	28° – 20°	2.34	0.40	2116	5.81	<0.001

371

372 c)

Experimental evolution regime	<i>Rejection rate per courtship</i>			
	<i>Effect</i>	<i>Chisq</i>	<i>Df</i>	<i>P value</i>

20±4°C	Temperature treatment	4.66	2	0.097
24±4°C	Temperature treatment	4.30	2	0.116
28±4°C	Temperature treatment	22.11	2	<0.001

373

374 d)

Experimental evolution regime	<i>Rejection rate per courtship</i>					
	<i>Contrast</i>	<i>Estimate</i>	<i>SE</i>	<i>Df₂</i>	<i>t-value</i>	<i>P value</i>
20±4°C	20° – 24°	0.76	0.40	2117	1.91	0.134
	28° – 24°	0.11	0.26	2117	0.43	0.900
	28° – 20°	-0.65	0.39	2117	-1.69	0.207
24±4°C	20° – 24°	0.04	0.29	2117	0.16	0.986
	28° – 24°	-0.28	0.21	2117	-1.34	0.375
	28° – 20°	-0.33	0.27	2117	-1.24	0.427
28±4°C	20° – 24°	1.56	0.35	2117	4.48	<0.001
	28° – 24°	0.22	0.16	2117	1.36	0.361
	28° – 20°	-1.34	0.34	2117	-3.91	<0.001

375

376 e)

<i>Rejection rate</i>			
<i>Effect</i>	<i>Chisq</i>	<i>Df</i>	<i>P value</i>
Experimental evolution regime x temperature treatment x mating system	7.279	4	0.122
Experimental evolution regime x mating system	0.926	2	0.629
Temperature treatment x mating system	1.040	2	0.594

377

378 **Table S8.** a) Summary statistics from fitting generalized linear mixed models for each temperature
 379 treatment due to significant interaction between temperature treatment and mating system for
 380 courtship rate. b) polyandry – monogamy contrast table from Tukey’s post hoc from the full model
 381 as an additional way to explore the interaction. c) statistical test of non-significant results.

382 a)

Temperature treatment	<i>Courtship rate</i>			
	<i>Effect</i>	<i>Chisq</i>	<i>Df</i>	<i>P value</i>
20°C	Mating system	2.34	1	0.125
24°C	Mating system	9.35	1	0.002
28°C	Mating system	54.40	1	<0.001

383
 384 b)

Temperature treatment	<i>Courtship rate</i>				
	<i>Estimate</i>	<i>SE</i>	<i>Df₂</i>	<i>t-value</i>	<i>P value</i>
20°C	-0.09	0.08	2110	-1.033	0.301
24°C	-0.20	0.08	2110	-2.520	0.012
28°C	-0.53	0.07	2110	-7.495	<0.001

385
 386 c)

<i>Courtship rate</i>			
<i>Effect</i>	<i>Chisq</i>	<i>Df</i>	<i>P value</i>
Experimental evolution regime x temperature treatment x mating system	1.369	4	0.849
Experimental evolution regime x mating system	1.023	2	0.599
Experimental evolution regime x temperature treatment	6.190	4	0.185

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391 **Table S9.** Estimates of the explained variance by the fixed effects only for all full models. For
 392 rejection and courtship rates, we ran zero-inflated models, and the explained variance was calculated
 393 accordingly. Similarly, for the rejection per courtship rate we ran a binomial model.

394

Variable modelled	Explained variance (R²m)
PC1	0.1483
Female reproductive success	0.0849
Lifespan	0.0358*
Rejection rate	delta (0.6106) lognormal (0.8928) trigamma (0.0965)
Rejection per courtship rate	theoretical (0.8773) delta(0.7642)
Courtship rate	delta (0.1101) lognormal (0.1275) trigamma (0.0924)

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*Using Pseudo-R²

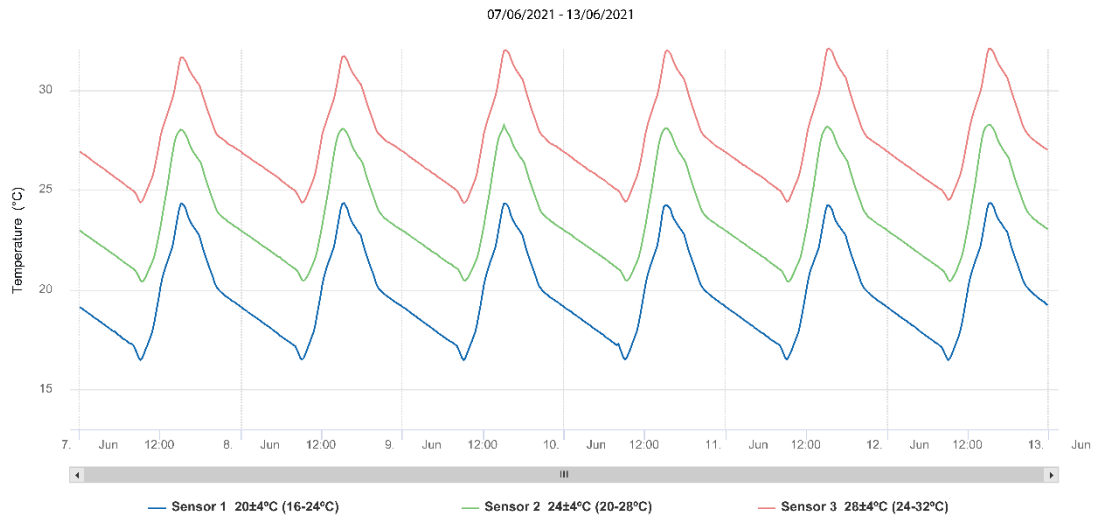
396 **Table S10.** Parameters used for Elastic net analysis.

397

Data set	α	λ
Virgin males (whole proteome)	0.5	0.68173
Mated males (whole proteome)	0.3	0.236615
Virgin males (SFPs)	0.2	0.681743
Mated males (SFPs)	0.1	0.700878

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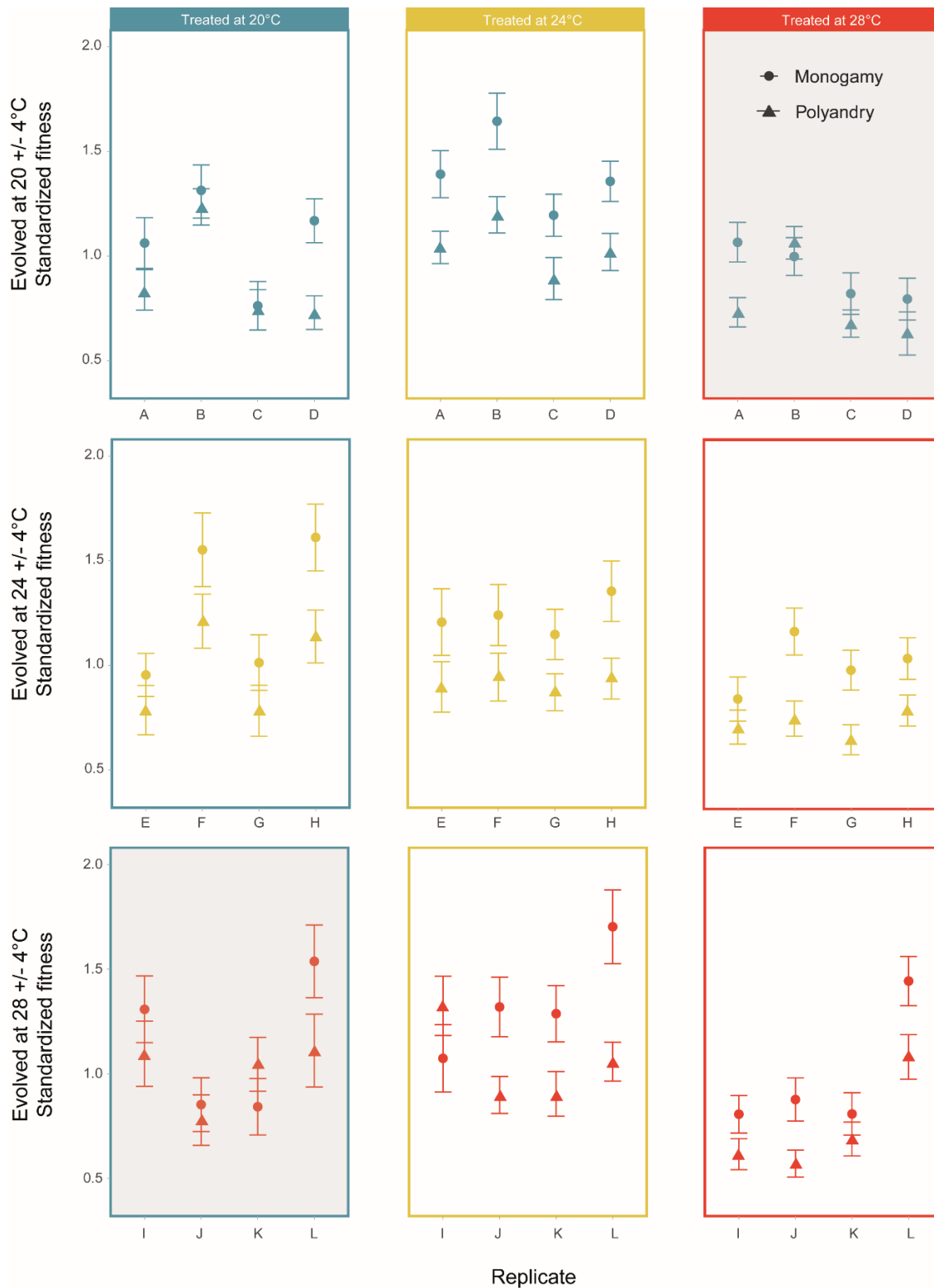
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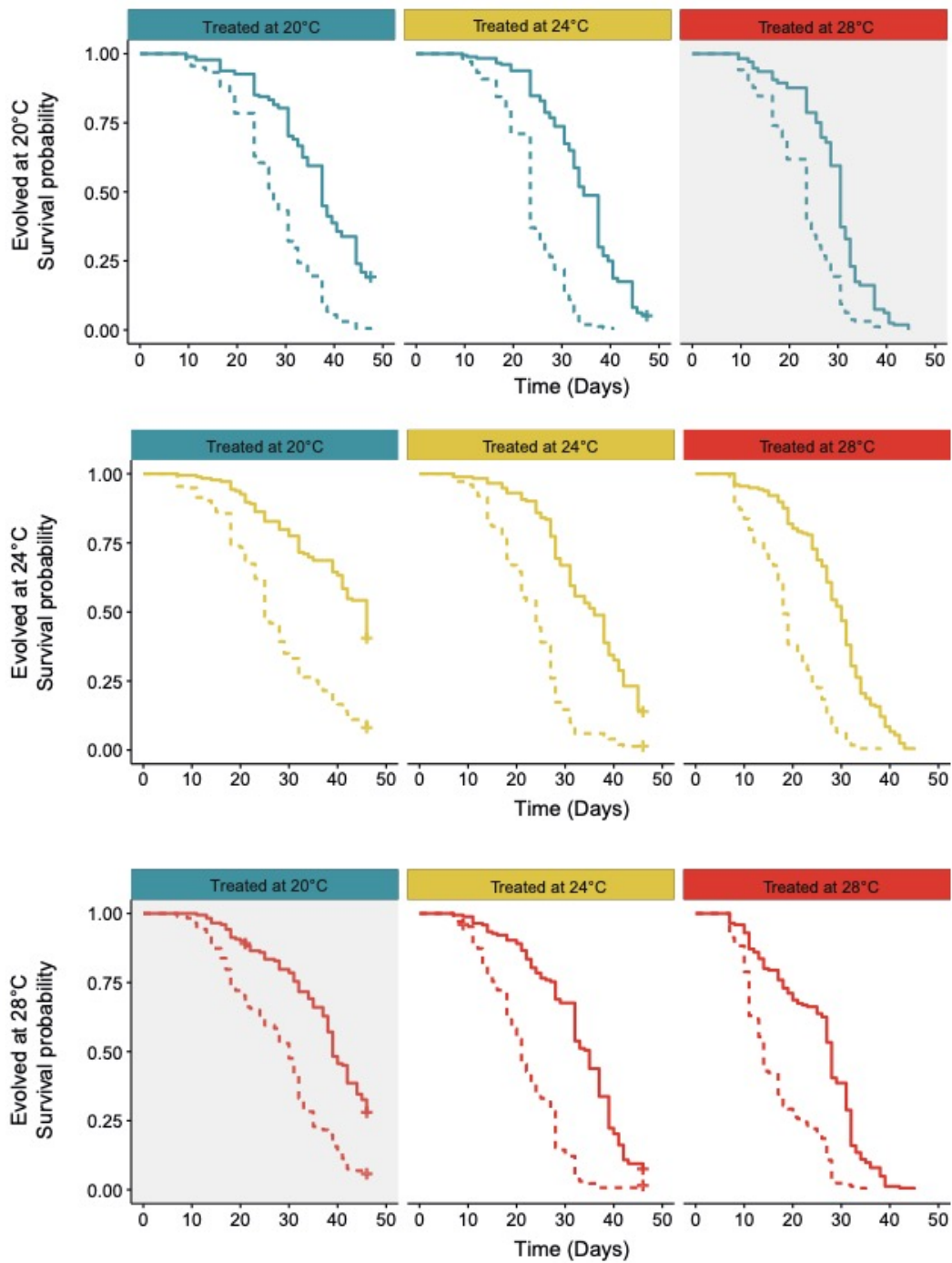
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402 **Figure S1 | Daily temperature variation profile for the experimental evolution regimes.** The
 403 regimes were based on average temperatures of 20°C, 24°C, or 28°C, with daily pre-programmed
 404 fluctuations of $\pm 4^\circ\text{C}$ to mimic natural circadian temperature variation. The blue line represents the
 405 cold thermal regime, the green line represents the moderate regime, and the red line represents the
 406 hot regime. Data correspond to temperature profiles recorded over a week in June 2021 as an example,
 407 using the extra sensors implemented to monitor environmental conditions during the experiment.

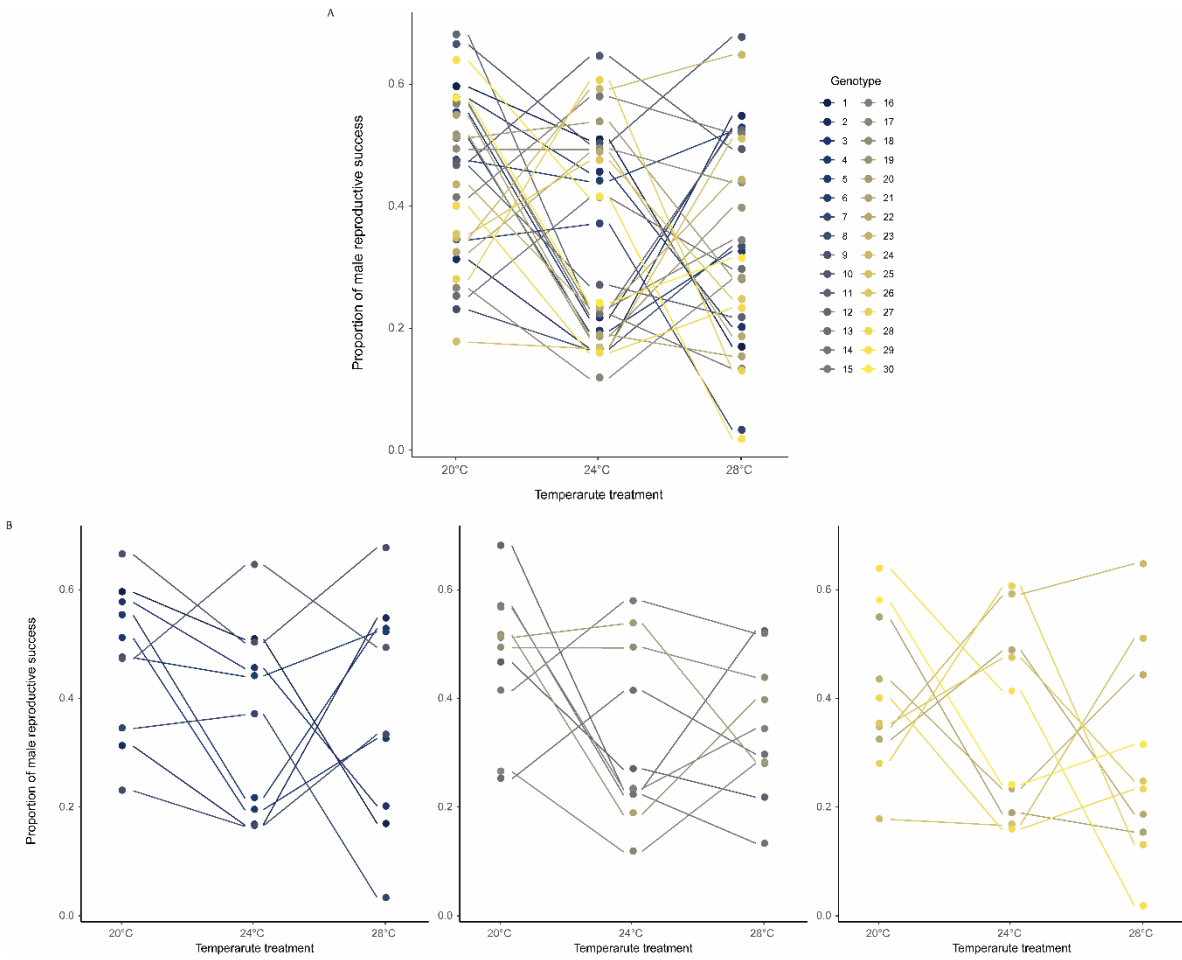


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Figure S2 | Effect of mating system, temperature treatment and experimental evolution regime on female fitness by replicate. Female reproductive success (mean \pm s.e.) across treatments and replicates. Data were standardized for each experimental evolution regime by dividing each value by the mean of the regime. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective regime.



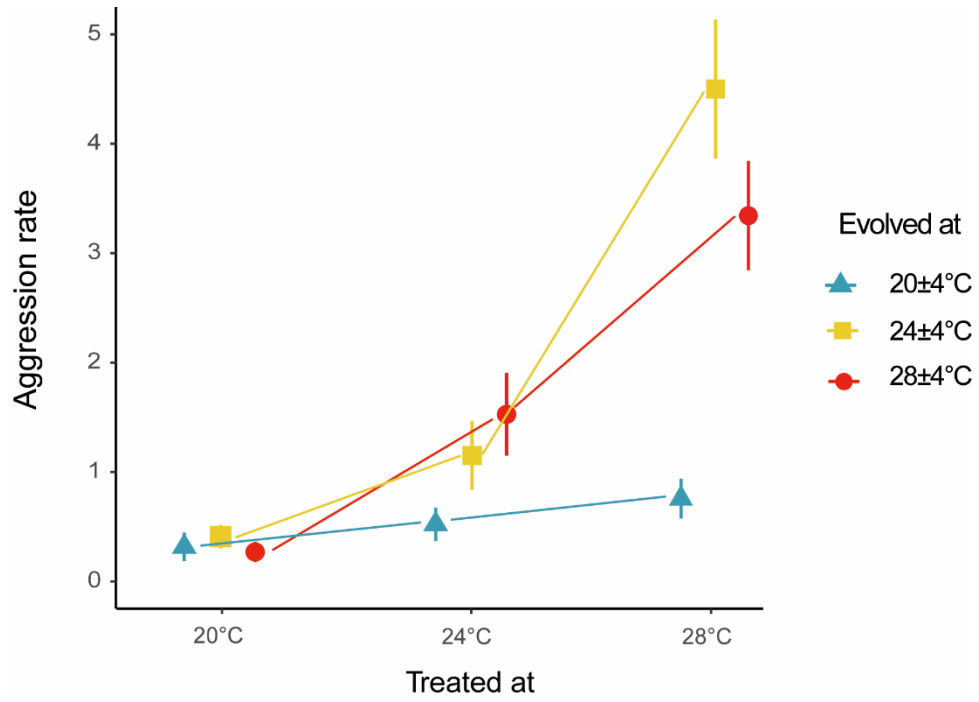
414
 415 **Figure S3 | Effect of mating system, temperature treatment and experimental evolution regime**
 416 **on female survival.** Female survival (four replicates) across mating systems, temperature treatments
 417 and experimental evolution thermal regimes. The difference between female survival kept in
 418 monogamy (solid lines) vs. polyandry (dashed lines), was higher when flies were treated at
 419 temperatures within the thermal regime of evolution, compared to those outside this range (shaded
 420 panels).
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425 **Figure S4 | Genotype-by-environment interactions for male reproductive success.** A) Reaction
426 norms for male reproductive success in 30 genotypes analysed across three temperature treatments.
427 B) Reaction norms for male reproductive success split into 3 panels to clearly illustrate each line.

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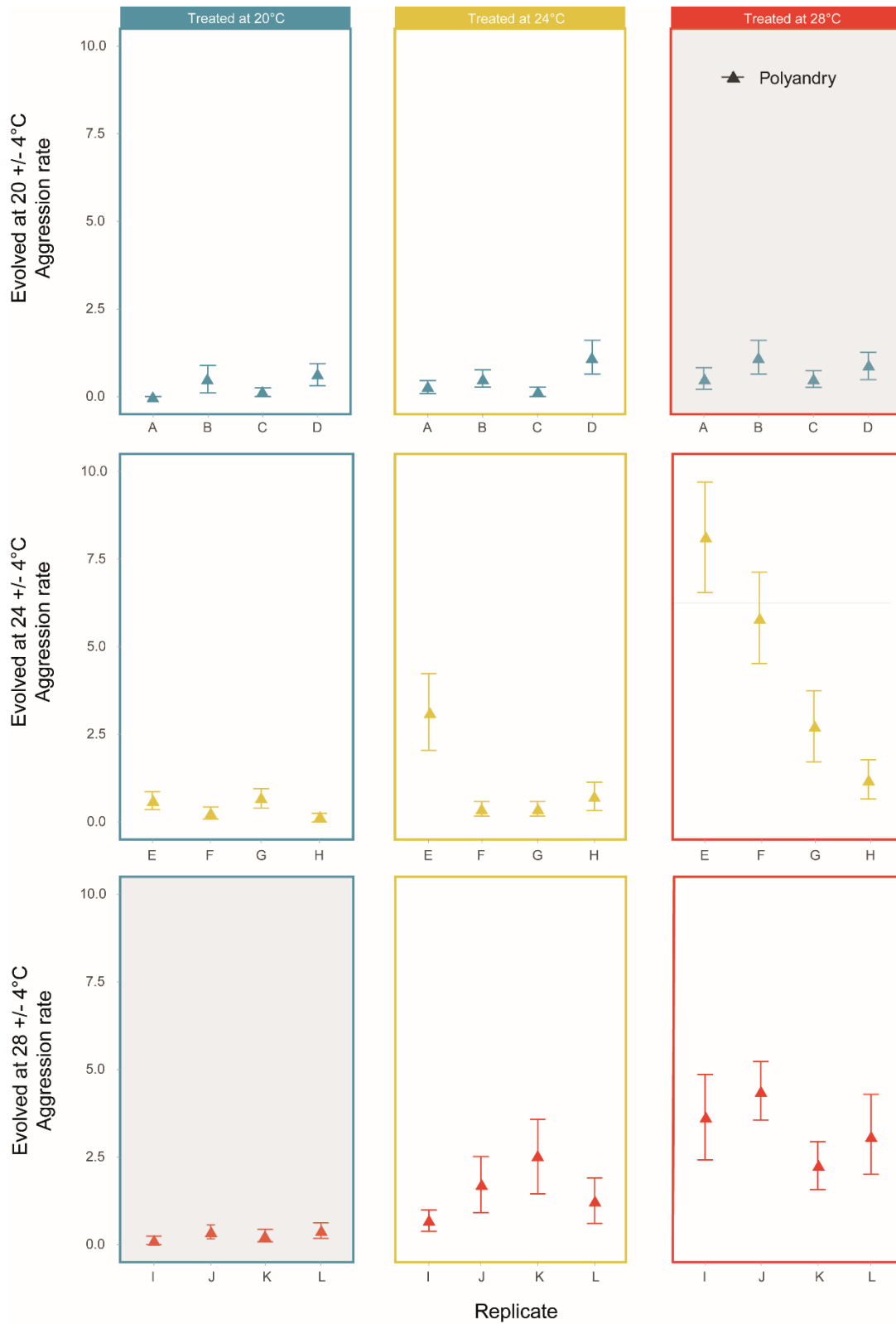
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432 **Figure S5 | Effect of temperature treatment and experimental evolution regime on male-male**
 433 **aggression rate.** Aggressions male-male per hour (mean ± s.e.; four replicates) across temperature
 434 treatments and experimental evolution thermal regimes.

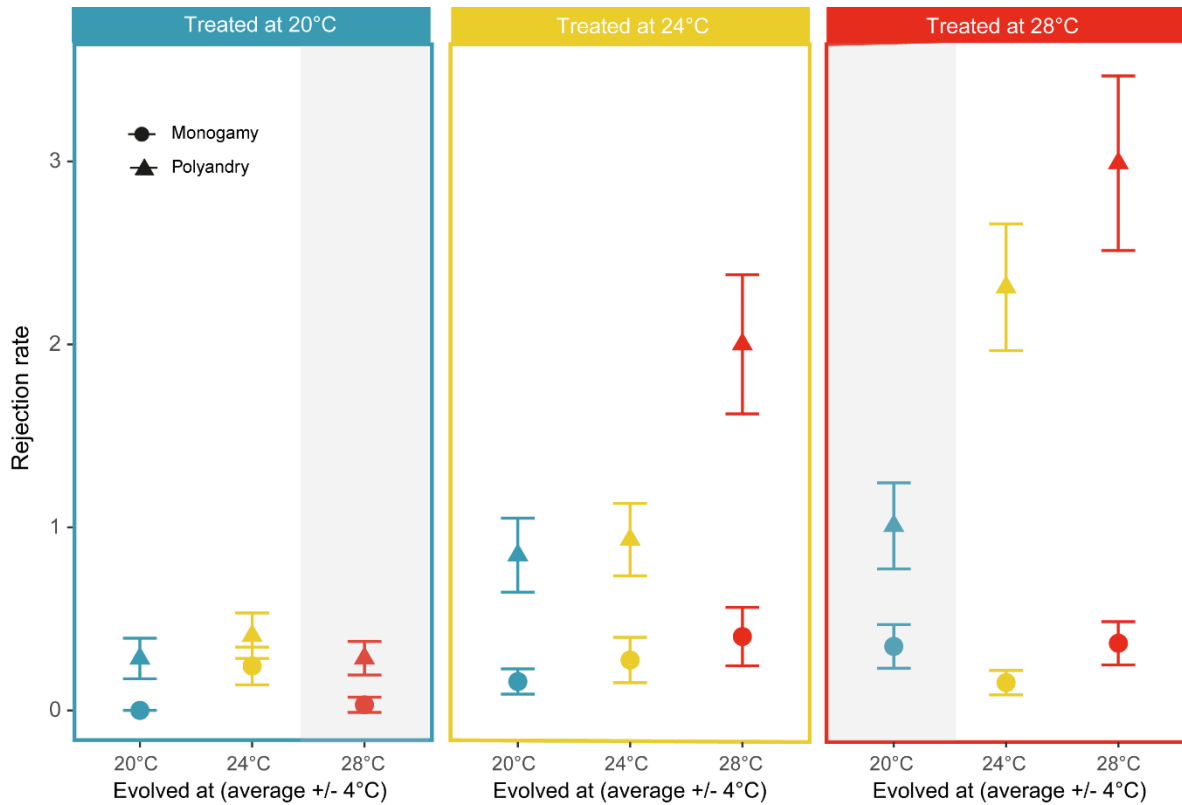
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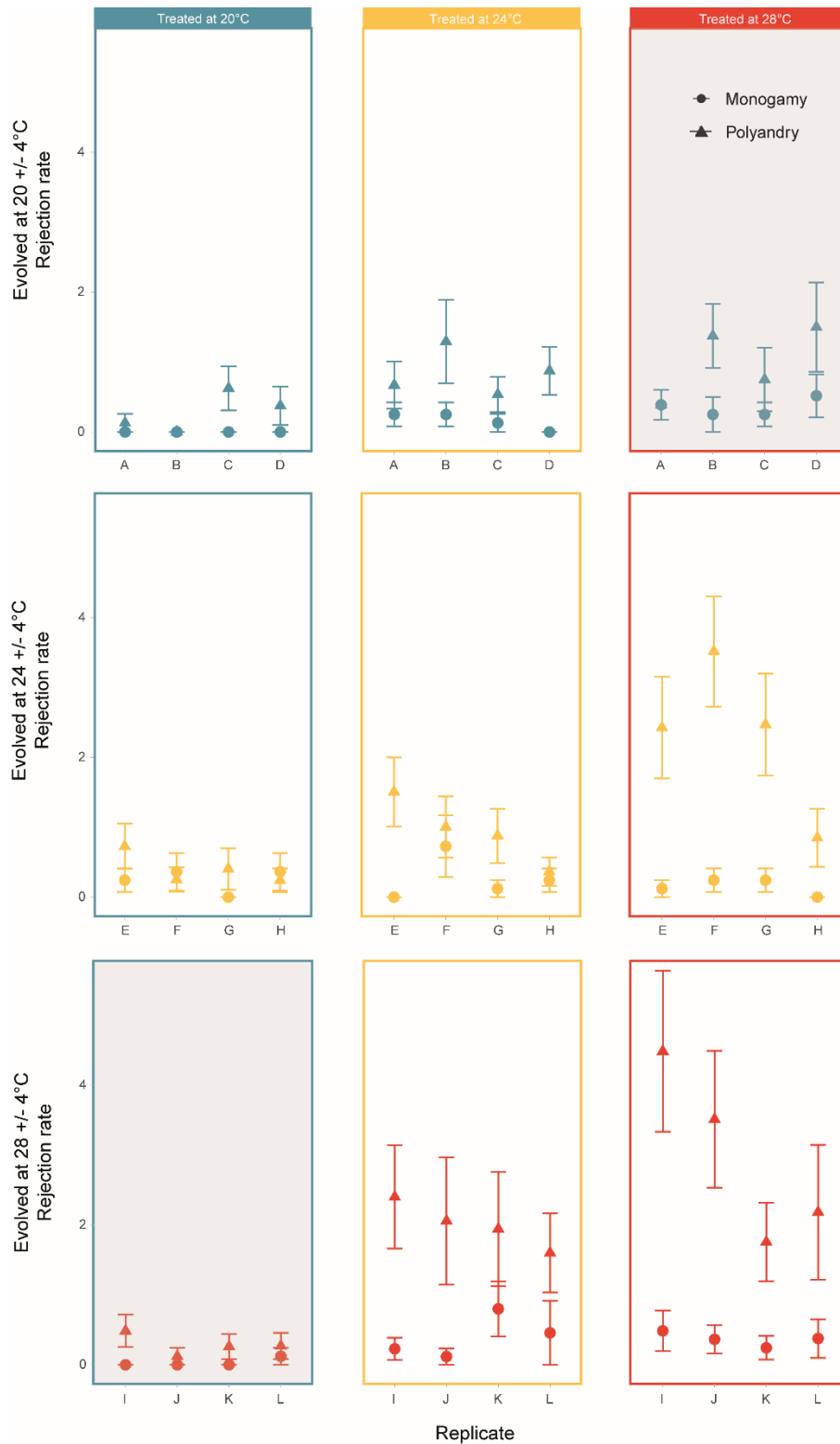
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Figure S6 | Effect of temperature treatment and experimental evolution regime on male-male aggression rate by replicate. Aggressions male-male per hour (mean ± s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective regime.



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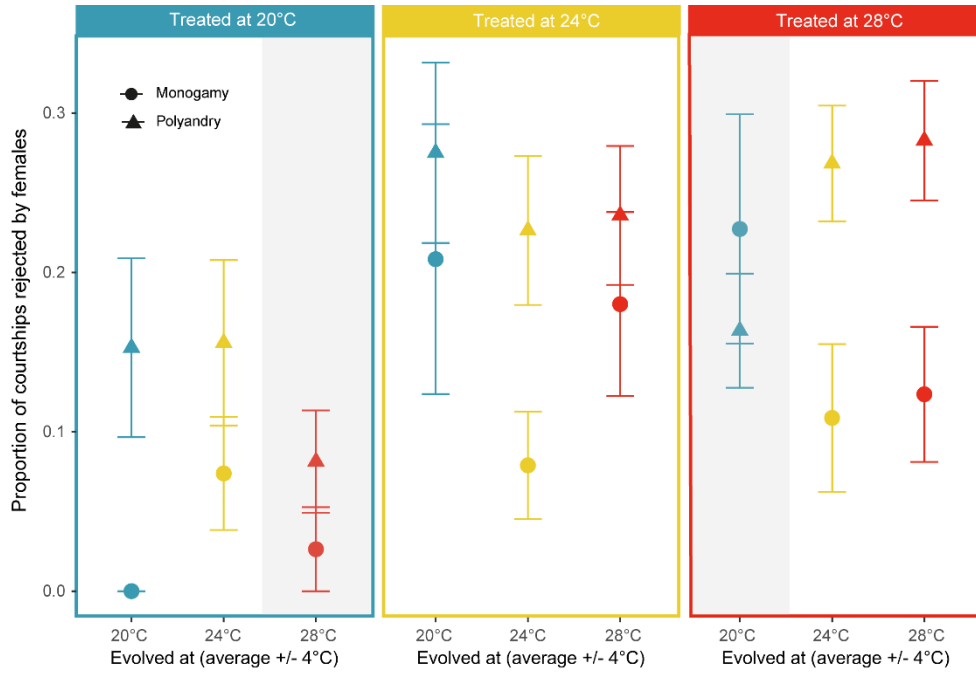
Figure S7 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate. Female rejections per hour (mean ± s.e.; four replicates) across temperature, mating system treatments and experimental evolution thermal regimes. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective regime.



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Figure S8 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate by replicate. Female rejections per hour (mean \pm s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective regime.

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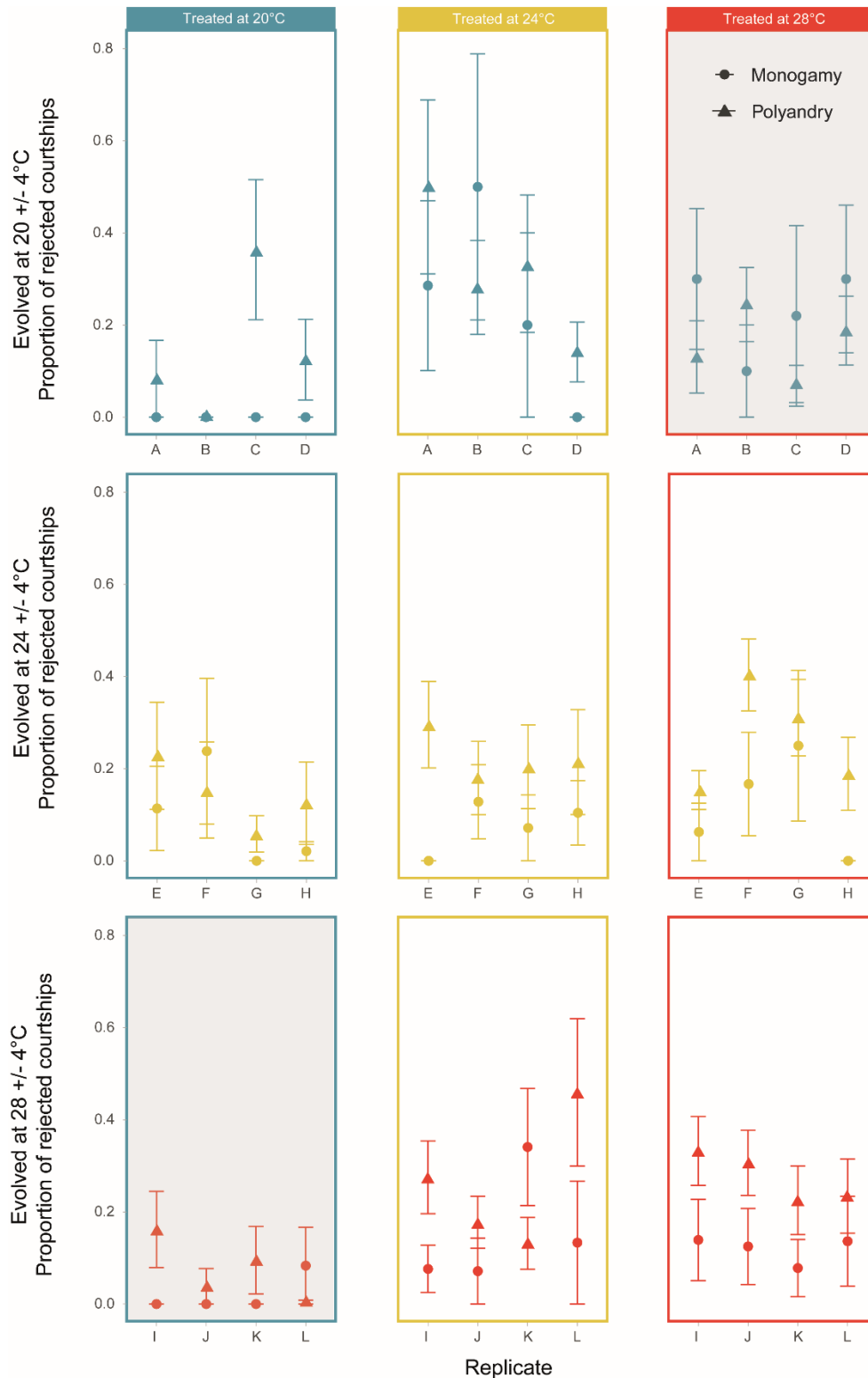
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461 **Figure S9 | Effect of mating system, temperature treatment and experimental evolution regime**
462 **on female rejection rate per courtship.** Proportion of courtships rejected by females (mean \pm s.e.;
463 four replicates) across temperature, mating system treatments and experimental evolution thermal
464 regimes. Shaded panels denoted temperature treatments outside the experimental evolution thermal
465 regime for the respective regime. Rejection rate per courtship was only calculated 30% of the
466 observation time (when courtship rate was different from 0).

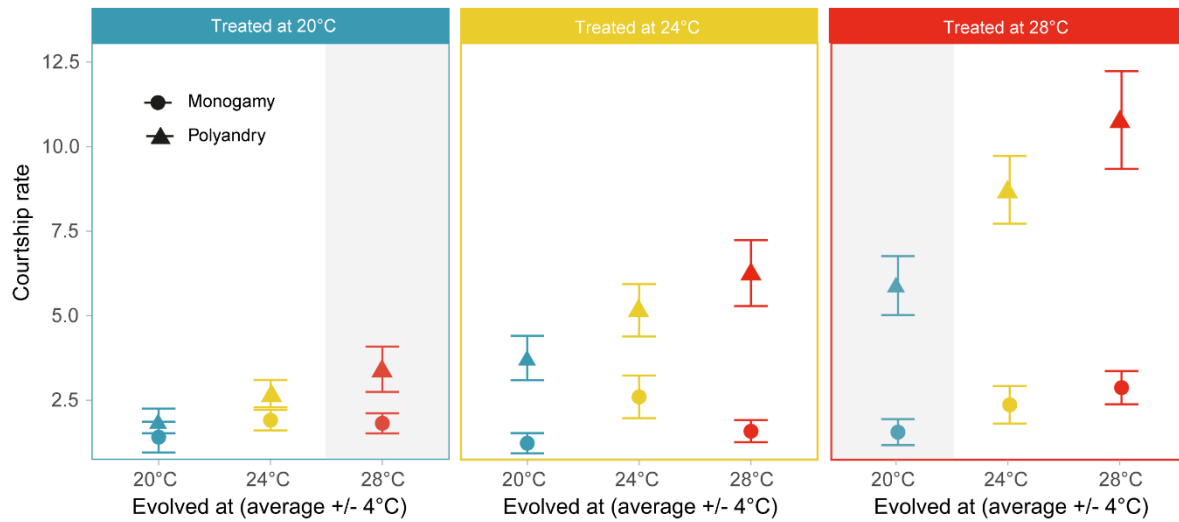
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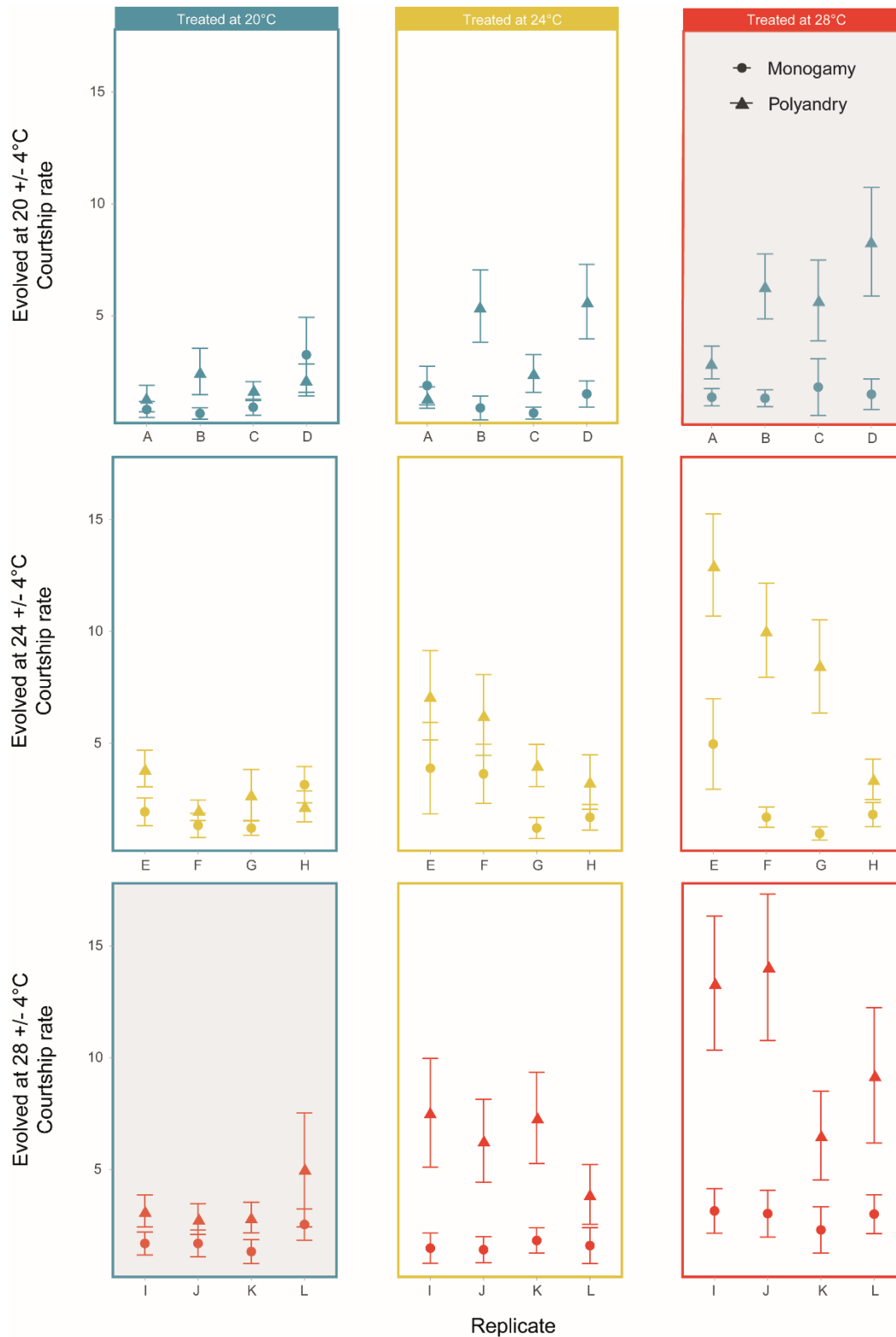
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Figure S10 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate per courtship by replicate. Proportion of courtships rejected by females (mean ± s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective regime. Rejection rate per courtship was only calculated 30% of the observation time (when courtship rate was different from 0).



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477 **Figure S11 | Effect of mating system, temperature treatment and experimental evolution regime**
 478 **on courtship rate.** Courtship per female per hour (mean \pm s.e.; four replicates) across temperature,
 479 mating system treatments and experimental evolution thermal regimes. Shaded panels denoted
 480 temperature treatments outside the experimental evolution thermal regime for the respective regime.
 481



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483 **Figure S12 | Effect of mating system, temperature treatment and experimental evolution regime**
 484 **on courtship rate by replicate.** Courtship per female per hour (mean ± s.e.) across treatments and
 485 replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal
 486 regime for the respective regime.