

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

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24

25 **ABSTRACT**

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

39

40 **Introduction**

41 Sexual selection can improve population viability and evolvability, making populations better
42 able to adapt to a changing environment (Cally et al., 2019; Lorch et al., 2003; Lumley et al.,
43 2015; Rowe & Houle, 1996). Driven by competition for mates and their gametes, sexual
44 selection is widespread and important in both females and males (Fromonteil et al., 2023).
45 Nevertheless, anisogamy commonly results in asymmetries in the strength and form of sexual
46 selection across the sexes (Janicke et al., 2016). Typically stronger sexual selection in males
47 allows for the effective purging of deleterious mutations and the capture of good genes
48 (condition-dependent genic capture) at a relatively cheap demographic cost, inasmuch
49 females are spared the brunt of selection (Cally et al., 2019; Lorch et al., 2003; Rowe &
50 Houle, 1996). However, the same divergent selective pressures that make sexual selection
51 such an effective evolutionary sieve also set the scene for sexual conflict, scenarios where
52 female and male evolutionary interests misalign (Parker, 1979). Alleles that confer a
53 reproductive advantage to one sex may have opposing effects in the other, leading to
54 reproductive strategies that evolve against each other (Pizzari & Snook, 2003; Rankin &
55 Kokko, 2006). Such sexually antagonistic coevolution is particularly salient in polygamous
56 species, where it frequently leads to adaptations in males that make them better competitors
57 in the sexual selection arena, but at the expense of harming females (Arnqvist & Rowe, 2005;
58 Chapman et al., 1995; Holland & Rice, 1999; Rice, 1996).

59

60 Harmful male adaptations to females (male harm) are incredibly pervasive, diverse
61 and sophisticated across the tree of life (Arnqvist & Rowe, 2005). On the one hand, male
62 harassment of females during pre-copulatory competition for mating has been documented in
63 many vertebrate and invertebrate species (Gómez-Llano et al., 2024). On the other, post-
64 copulatory competition has given rise to male harm adaptations that are similarly widespread

65 and far more complex, ranging from toxic ejaculates (Wigby & Chapman, 2005) to
66 adaptations for traumatic insemination (Crudginton & Siva-Jothy, 2000; Koene &
67 Schulenburg, 2005; Lange et al., 2013). Male harm thus drives antagonistic female-male co-
68 evolution in a host of behavioural and morphological traits (Arnqvist & Rowe, 2005), and
69 may even act as an engine of speciation (Gavrilets, 2014; Rice et al., 2005). More
70 importantly, male harm frequently leads to a “reproductive tragedy of the commons” where
71 selection on male fitness impacts population demography by depressing net female
72 productivity (Gómez-Llano et al., 2024), even to the point of facilitating extinction (Le
73 Galliard et al., 2005). Recent theoretical models suggest that such negative effects may
74 compound when harmful traits are linked to condition (Pitnick & Garcia-Gonzalez, 2002), in
75 which case good-genes selection can be counteracted by male harm (Flintham et al., 2023)
76 and male harm can slow down evolutionary rescue (Gómez-Llano et al., 2024). In short,
77 sexual selection acts as a double-edge sword for populations because stronger condition-
78 dependent selection on males, which allows for the demographically cheap purging of
79 deleterious alleles, the genic capture of good genes, and ensuing fast adaptation, frequently
80 turns out to be a recipe for intense sexual conflict. Disentangling what, then, determines
81 whether strong sexual selection and ensuing conflict leads to harm to females and what shape
82 it takes, its diversity in form and intensity, is a main concern in evolutionary biology.

83

84 A surge of studies point towards ecology as a way to better understand the evolution
85 of male harm and its consequences for populations (Perry & Rowe, 2018). Ecology has been
86 shown to play a central role in shaping patterns of population divergence via sexual conflict
87 (Arbuthnott et al., 2014; Perry et al., 2017), as well as in determining the intensity of male
88 harm and to what degree it may offset good genes selection (Londoño-Nieto et al., 2023; Yun
89 et al., 2017, 2018). Temperature is a particularly interesting ecological factor to this respect,

90 as it modulates a wide range of physiological, morphological and behavioural traits,
91 impacting individuals and populations at a global taxonomic scale. Furthermore, temperature
92 exhibits marked spatio-temporal variation, such that for most species in the wild competition
93 over reproduction (and consequently male harm) will unfold in a dynamic thermal
94 environment. This is being taken to the extreme by the current global warming crisis.
95 Importantly, recent research in *Drosophila melanogaster* shows that both the intensity of
96 male harm and its impact on different female fitness components are very thermally plastic
97 (García-Roa et al., 2019; Londoño-Nieto et al., 2023), suggesting that temperature may be a
98 key player in male harm evolution (García-Roa et al., 2020).

99

100 To test this idea, we collected *D. melanogaster* from a population that has been shown
101 to be thermally plastic for male harm (Londoño-Nieto et al., 2023) and set up 12
102 experimental evolution lines under three different thermal regimes mimicking natural
103 seasonal and circadian temperature variation. *D. melanogaster* is a model species in the study
104 of sexual conflict and has well-characterized pre- and copulatory male harm mechanisms.
105 During male-male pre-copulatory competition, males harm females via intense harassment
106 that causes substantial costs in the form of physical injuries and energetic/opportunity costs
107 (Bretman & Fricke, 2019; Partridge & Fowler, 1990; Teseo et al., 2016). In the context of
108 sperm competition, male seminal fluid proteins (SFPs) manipulate female re-mating and egg-
109 laying rates to the male's advantage, but frequently at a cost to female fitness (Chapman et
110 al., 2003; Wigby & Chapman, 2005). These proteins are secreted by male accessory glands
111 and are strategically allocated by males in response to even subtle variations in the socio-
112 sexual context (Hopkins et al., 2019a, b; Sirot et al., 2011), suggesting they are sensitive to
113 environmental variation. After 29-30 generations of experimental evolution, we subjected all
114 populations to two generations at $24\pm 4^{\circ}\text{C}$ (to erase parental/grand-parental effects) and then

115 set up a series of fitness, behavioural and seminal proteome assays to measure experimental
116 evolution effects on: male harm intensity (i.e. how much male-male competition depresses
117 female fitness), the thermal plasticity of such effects, and its underlying pre- (male aggression
118 and harassment levels) and copulatory (SFPs) mechanisms. Our aim was to examine whether
119 adaptation to different temperatures determines overall male harm levels, its form (e.g.
120 relative importance of harassment vs. seminal toxicity), and its thermal plasticity, thus
121 gaining insight into the factors governing male harm evolution and the impact of changing
122 temperatures (e.g. global warming) on sexual conflict and the evolution of the male seminal
123 proteome.

124

125 **Methods**

126 Experimental evolution design

127 12 experimental populations, each with a controlled size of 100 males and 100 females, were
128 established from our stock field-collected population “Vegalibre” (see Londoño-Nieto et al.,
129 2023). Populations (4 replicates per treatment) evolved under one of three temperature
130 regimes: average of 20, 24 or 28°C with daily pre-programmed fluctuations of $\pm 4^\circ\text{C}$ that
131 mimic circadian temperature variation, at ~60% humidity, and on a 12:12 hr light:dark cycle.
132 Populations were maintained in non-overlapping generations to control for population size.
133 Each generation began by releasing 100 randomly selected same-aged males and females (N
134 = 200) into a glass (16.5 x 19.5cm) bottle with two bottles with 75ml of standard food
135 (Londoño-Nieto et al., 2023). We allowed 6 days of interaction, collecting eggs on the 6th day
136 that we raised at a standardized density (Clancy, D. J., & Kennington, 2001) in bottles with
137 75ml standard food. We isolated emerging virgins from these bottles in same-sex vials and
138 used them to setup the next generation when 3-4d old. This design selected for early
139 reproduction, so that cumulative harm effects over time are unimportant for females and thus

140 selection for female resistance should be minimized (Bonduriansky et al., 2008; Filice et al.,
141 2020). Populations were assayed after 29-30 generations of experimental evolution and two
142 generations of common garden at $24\pm 4^{\circ}\text{C}$ to control for parental and grand-parental effects.
143 Experimental evolution started in February 2020 for all lines, and finished in August 2021 for
144 the hot regime, in October 2021 for the moderate regime and in April 2022 for the cold
145 regime. Differences in evolution time are due to differences in development time at each
146 thermal regime. Populations from the hot regime were assayed between September and
147 October 2021, from the moderate regime between November and December 2021 and from
148 the cold regime between June and July 2022.

149 Male harm and behavioural assays (experiment 1)

150 To examine the effect of thermal evolution regimes on overall male harm levels and its
151 thermal plasticity, we used the standard procedure of comparing reproductive success and
152 survival of experimentally evolved female flies from each population under monogamy (low
153 sexual conflict; one female and one male per vial) and polygamy (high sexual conflict; one
154 female and three males per vial). This is standard procedure to gauge male harm in
155 *Drosophila*, where these sex ratios represent biologically relevant scenarios (Dukas, 2020;
156 Gómez-Llano et al., 2024; Yun et al., 2021). For each experimental evolution line, we
157 replicated these assays at 20, 24, and 28°C for six weeks. We collected experimental flies as
158 virgins, isolated them into same-sex vials of 15 individuals and then randomly allocated them
159 to either of the three temperature treatments, 48 hours before starting the experiment. Flies
160 remained at those temperatures until the end of the assay. To begin the experiment, we placed
161 virgin focal females (4-5d old) in individual vials containing medium supplemented with live
162 yeast, after which we immediately added one (monogamy) or three (polygamy) experimental
163 males from the corresponding population to each female vial. During the day 1 of the
164 experiment, we observed flies (120 vials per treatment, 30 per replicate) for 8 h using a

165 combination of scan sampling and all-occurrences recording rule to score courtship intensity
166 (number of courting males per female per hour), male-male aggression rate (number of
167 aggressions per hour) and female rejection rate (number of rejections per hour; see Bastock &
168 Manning, 1955; Connolly & Cook, 1973) to investigate whether pre-copulatory male harm
169 mechanisms, and their thermal plasticity, were affected by experimental evolution. To
170 estimate female reproductive success, we transferred flies to fresh vials twice a week and
171 incubated the vials containing the eggs from focal female at $24\pm 4^{\circ}\text{C}$ for 15-20 d (~15 d for
172 vials coming from 28°C , ~17 d for 24°C and ~20 d for 20°C) to allow F1 offspring
173 emergence, after which we froze them at -21°C for later counting. Differences in incubation
174 time are due to differences in developmental temperature during the first 1-4 days (depending
175 on when individual eggs were laid in relation to when vials were flipped). We discarded and
176 replaced males with young (2-4d old) virgin males (same treatment as described above) three
177 weeks after starting the experiment. We kept male and female flies under these conditions for
178 six weeks, after which we discarded all of them. We recorded survivorship of focal females
179 daily and replaced dead male flies if needed with stock replacement males maintained at each
180 of the temperature treatments. Samples sizes are provided in the Table 1.

181 We modelled reproductive success as the response variable in a linear mixed model
182 (LMM), and courtship, male-male aggression and female rejection rates as the response
183 variables in generalized linear mixed models (GLMM) with experimental evolution regime,
184 temperature treatment, mating system and their interactions as fixed effects, and replicate
185 population as a random effect using *lme4* (Bates et al., 2015) and *glmmTMB* (Brooks et al.,
186 2017) packages in RStudio (version 4.2.2). We modelled survivorship as the response
187 variable in a Cox proportional hazard model with the same fixed and random effects using
188 *coxme* and *survminer* packages (Kassambara & Kosinski, 2018; Therneau, 2022). For male
189 harm via harassment, previous studies in this species have shown that it is directly related to

190 courtship intensity, female rejection and male intrasexual competition via male-male
191 aggression (Bretman & Fricke, 2019; Carazo et al., 2014; Partridge & Fowler, 1990). To
192 study the joint contribution of these behaviours, we also modelled component 1 of a PCA that
193 explained 67.7% of the variation in the data, whereby male-male aggression, courtship
194 intensity and female rejection all loaded in the same direction (Table S1a), so we took PC1 as
195 an overall index of male harassment to females. As our replicates are from different
196 populations, we also fitted random slopes models for correlated fixed effects of temperature
197 evolution regime and temperature treatment (Arnqvist, 2020). However, in all cases we found
198 that fixed slopes models presented the minimum AICc value, supporting them as the best
199 models given the trade-off between fit to the data and model complexity (Konishi &
200 Kitagawa, 2008); but we note results did not change qualitatively in either case. We
201 performed model selection by backward stepwise elimination; refitting models without the
202 triple interaction where necessary to arrive at the minimal adequate model. Replicate
203 population was kept on all analyses to control for this variation.

204 Additionally, to specifically explore if overall harm was higher at adaptive
205 temperatures, we run extra models to compare female fitness at adapted vs. non-adapted
206 temperatures in flies from cold and hot experimental evolution regimes (i.e., flies from the
207 moderate regime were not assayed in maladaptive temperatures). We modelled experimental
208 evolution regime, mating system and adaptive temperature (factor with two levels, yes/no)
209 and the interaction between mating system and adaptive temperature as fixed effects, and
210 replicate population as random effect. When we detected a significant interaction between
211 main effects, we ran models separately for each evolutionary temperature regime or
212 temperature treatment to explore the nature of such interactions. We also run post hoc
213 Tukey's test as an additional way to explore interactions while controlling for inflation of

214 experiment-wise type 1 error rate. We assessed significance with *F* test for LMM and
215 chisquare test for GLMM and Cox proportional hazard models.

216 Proteomics assays (experiment 2)

217 To study whether and how the seminal fluid of males evolves in response to temperature, we
218 set up a series of assays and conducted label-free quantitative proteome analysis of the
219 accessory glands of mated and virgin males across experimentally evolved lines. All assays
220 were conducted at the common garden temperature of 24°C, which was the shared
221 temperature in all three thermal evolution regimes. Upon eclosion, we allocated virgin focal
222 males into vials of 8 individuals in which they aged for 4-5 days. On the day of sample
223 collection, we isolated 45 experimental females per population in yeasted vials, after which
224 we immediately introduced focal males either into a female-containing vial or into an empty,
225 yeasted vial to be retained as a virgin. We flash frozen the mated males in liquid nitrogen 25
226 min after the start of mating, freezing a virgin male from the same population at the same
227 time. Freezing males at 25 min after the start of mating ensure a complete mating and is
228 consistent with the protocol used previously for proteomics experiments (Hopkins et al.,
229 2019a, b; Sepil et al., 2019). We repeated this procedure during two more consecutive days to
230 obtain three independent biological replicates. Thus, populations from the same evolutionary
231 temperature regime were assayed during the same three consecutive days. We stored all
232 frozen samples at -80°C until dissection, for which we thawed flash frozen males and
233 dissected their accessory glands on ice in phosphate-buffered saline (PBS) buffer, under a
234 Leica M80 binocular scope. Each biological replicate (i.e., sample) consisted in a pool of 20
235 reproductive glands from males evolved at the same temperature regime, of the same mating
236 status (virgin or mated) and of the same replicate in 25- μ l PBS buffer on ice, which we sent
237 for label-free quantitative proteomics sample preparation and quantification at the SCISIE
238 proteomics service at the University of Valencia. Hence, we had six samples per population

239 (three from virgin and three from mated males), and four populations per each evolutionary
240 temperature regime. In total, we had 72 samples (24 from each of the three experimental
241 evolution treatments). Our quantitative proteomics analysis was conducted in accordance
242 with the sample preparation protocol SWATH-MS (Gillet et al., 2012). Details of this
243 method, the LC-MS/MS platform, and the data processing are given in SI Appendix.

244 We conducted all proteomics analysis on normalized abundances. We normalized the
245 protein areas calculated by the total sum of the areas of all the quantified proteins. We
246 generated two different data sets to analyse our proteomics data. One included all samples
247 from virgin males and another one included all samples from mated males. We used an
248 elastic net penalized logistic regression model to analyse our data sets, using *glmnet*
249 (Friedman et al., 2010) package in RStudio. The elastic net regression is a hybrid technical
250 least square regression method that involves regularization and variable selection and is
251 particularly useful when the number of predictors is much bigger than the number of
252 observations (Zou & Hastie, 2005). We also analysed our data sets using tests of reduction of
253 dimensionality PLS-DA, using *mixOmics* (Rohart et al., 2017) package in RStudio. For our
254 analysis and visualization of abundance patterns we took an average across three biological
255 replicates for each protein, population, experimental evolution regime and mating status. For
256 visualization, we used a Euclidean correlation distance metric and plotted the output as a
257 heatmap using the function *aheatmap* included in the *NMF* package (Gaujoux & Seoighe,
258 2010). Finally, we also identified all proteins described as seminal fluid proteins (SFPs),
259 based on a high-confidence SFPs reference list from Sepil et al., 2019 and Wigby et al., 2020.
260 For the virgin male and mated male datasets we represented the number of proteins and the
261 percentage of SFPs expressed in each evolutionary temperature treatment through Venn
262 diagrams using *ggvenn* package (Gao et al., 2021) in RStudio.

263 GxE assay (experiment 3)

264 Finally, to test for the existence of GxE interactions within the range of temperatures at
265 which reproduction is optimal for the ancestral wild population (20-28°C) of our focal flies,
266 we conducted a series of fitness assays across 30 male genotypes (i.e. isogenic lines) derived
267 from wild-caught flies from this wild population. We established isolines through 10
268 generations of inbreeding, resulting in flies sharing at least 96% of their genome (Falconer,
269 1996). Before the start of the experiment, we isolated 40 females per isoline into embryo egg-
270 laying cages with yeasted grape juice agar plates (FlyStuff grape agar premix, Genesee
271 Scientific), from which we collected experimental virgin wild-type (*wt*) male flies that we
272 placed into same-sex vials of 15 individuals. We used *sparkling^{poliert}* (*spa^{pol}*) backcrossed into
273 the Vegalibre population (i.e. same genetic background) as rival males and reproductive
274 females, a recessive phenotypic marker that can be used for paternity assignment. To begin
275 the experiment, we placed *wt* males from each isoline in individual vials containing medium,
276 after which we added two *spa^{pol}* males and one female, ensuring a high-competition
277 environment (i.e. three males competing over access to one female in a single vial). We then
278 placed four replicates (i.e., vials) per isoline under three different treatment temperatures (20,
279 24, and 28°C) with daily fluctuations ($\pm 4^\circ\text{C}$). Because we did not have enough flies to set up
280 four replicates in 6 isolines (see Data), we ended up with 342 replicas (114 per temperature
281 treatment). We replaced *spa^{pol}* females every two weeks and *spa^{pol}* males every four weeks,
282 so that focal males competed over access to different females against different males during
283 their lifespan, as happens in nature. We recorded survivorship and offspring production
284 following the same protocols as described for experiment 1. We calculated reproductive
285 success of focal males as the proportion of sired offspring vs. total offspring (*wt* + *spa^{pol}*), and
286 modelled it as the response variable in a GLMM using a Beta regression model (Smithson &
287 Verkuilen, 2006), with temperature as a fixed effect and isoline and their interaction as
288 random effects (Bolker et al., 2009) using *glmmTMB* (Brooks et al., 2017). We used

289 Nakagawa's R-squared (Nakagawa & Schielzeth, 2013) to extract the variance explained by
290 each model, analysed random effects using *ranef* function from lme4 (Bates et al., 2015), and
291 tested via likelihood ratio tests for significance.

292

293 **Results**

294 *Harm to females is higher at evolved temperatures*

295 For experiment 1 we found that experimental evolution significantly modulated the degree to
296 which increased conflict hampered female reproductive success (experimental evolution
297 regime x mating system interaction: $F_{2,2939.1} = 3.04$, $P = 0.048$), with higher male harm in flies
298 from the moderate experimental evolution lines (Figs. 1 and S1). This was driven mainly by
299 the fact that male harm was more constant (less plastic) in flies evolved in the moderate
300 thermal regime (experimental evolution regime x temperature treatment interaction: $F_{4,2939.5} =$
301 2.89 , $P = 0.021$), whereby the decrease in female reproductive success at high conflict was
302 lower at 28°C in flies evolved in the cold thermal regime and at 20°C in flies evolved in the
303 hot thermal regime (i.e. their respective non-adapted temperatures; Fig. 1 and Table S2).
304 Effects on female survival closely mimicked effects on female reproductive success
305 (experimental evolution regime x mating system interaction: $X^2_2 = 8.30$, $P = 0.016$;
306 experimental evolution regime x temperature treatment interaction: $X^2_4 = 55.92$, $P < 0.001$;
307 Fig. S2 and Table S3a, b). Finally, direct comparison of male harm levels between adapted
308 vs. non-adapted temperatures confirmed that, overall, male harm was higher at temperatures
309 within vs. outside the thermal range in which flies were allowed to evolve (adaptive
310 temperature x mating system interaction for female reproductive success, $F_{1,1942.1} = 4.12$, $P =$
311 0.042 , and survival, $X^2_1 = 10.89$, $P < 0.001$; see Figs. 1 and S2).

312 *Cold temperatures decrease the levels and thermal plasticity of male harassment*

313 Experimental evolution regime had clear effects on both overall male harassment and its
314 plasticity. Overall, harassment was lower ($F_{2,9} = 3.87$, $P = 0.06$) and less plastic
315 (experimental evolution x temperature treatment: $F_{4,1039.1} = 2.95$, $P = 0.019$; Fig. 2 and Table
316 S1b,c) in flies evolved in the cold thermal regime . Analysing these three behaviours
317 separately, as well as effects across mating systems, confirmed these results (see SI).

318 *The male seminal proteome evolves differently across thermal regimes, with SFPs*
319 *responding differentially at hot temperatures*

320 For experiment 2, we found a total of 1453 proteins, 148 of which have been priorly
321 identified as SFPs. We analysed virgin and mated data sets independently with the aim of
322 understanding how evolution affects the proteome of males that are competing for females
323 for the first vs. successive matings. For virgin males, 87 proteins were selected as predictor
324 variables with a strong effect on proteome quantification, 13 of which are known SFPs.
325 Euclidean distance correlation identified three different clusters for these 87 proteins, which
326 coincide with the three experimental evolution thermal regimes (Fig. 3a), with each cluster
327 including the four replicates within each regime. A partial least-squares discriminant analysis
328 (PLS-DA) supported these findings (Fig. 3b), showing that the overall composition of the
329 seminal fluid proteome responded differently to evolution at cold, moderate and hot thermal
330 regimes. We identified 11 and 41 proteins that were singularly over and under-expressed,
331 respectively, by males that evolved in hot vs. moderate/cold regimes, eight and seven that
332 were over and under-expressed by males that evolved in moderate vs. hot/cold regimes, and
333 nine and six that were singularly over and under-expressed by males that evolved in cold vs.
334 moderate/hot regimes (Fig.3c). While 72.7% of the proteins differentially over-expressed in
335 flies from the hot regime have been previously identified as SFPs, none of the differentially
336 overexpressed proteins at either of the two other regimes are known SFPs (Fig. 3c and Table

337 S4). We found over-expression of the SFP “Semp1” (protein ID Q9VJN9) by males evolved
338 in the hot regimen. This protein has been described to be transferred to females during mating
339 and it is necessary to process two other seminal proteins: the ovulation hormone ovulin and
340 the sperm storage protein in mated females (LaFlamme et al., 2014; Ravi Ram et al., 2006).

341 Results from mated males closely resembled the above results. Elastic net regression
342 identified 89 proteins as predictor variables with a strong effect on proteome quantification in
343 mated males, 15 of which have been previously identified as SFPs. According to the
344 abundance of those proteins, we again identified the same three different clusters that
345 coincide with the three experimental evolution regimes (Fig. 4a), which was also confirmed
346 by the PLS-DA analysis (Fig. 4b). 14 and 21 proteins were differentially over and under-
347 expressed, respectively, by males evolved in the hot regime. 12 and seven were differentially
348 over and under-expressed by males evolved in the moderate regime, and nine and 18 were
349 differentially over and under-expressed by males evolved in the cold regime. Six of the
350 proteins over-expressed by males evolved in hot regime are known SFPs (42.9%). As in
351 virgin males, none of the proteins differentially over-expressed at either of the two other
352 thermal regimes correspond to previously identified SFPs (Fig 4c and Table S4). Overall,
353 these results show clear responses of the male seminal proteome to experimental evolution at
354 different thermal regimes, suggesting that local adaptation to the warm regime is
355 characterized by the overexpression of SFPs.

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

357 For experiment 3, we found clear thermal GxE interactions for male reproductive success
358 ($X^2_{10} = 4.26$, $P < 0.001$), where the two most prevalent reaction norms reflected male
359 genotypes that had higher reproductive success either at moderate vs. hot/cold temperatures
360 (negative quadratic) or at hot/cold vs. moderate temperatures (positive quadratic; see Fig. 5).

361 **Discussion**

362 A central question in evolutionary biology is to understand what factors shape the evolution
363 of sexual conflict and male harm, its underlying mechanisms, and its net consequences for
364 populations. Here, we combined experimental evolution with behavioural, fitness and
365 proteomic assays in *Drosophila melanogaster* originating from a wild population to show
366 that thermal ecology can drive the evolution and diversification of pre-copulatory and
367 copulatory sexual conflict traits and resulting male harm to females. Our results show that
368 temperature might be key to unravel the evolution of sexual conflict and its underlying
369 mechanisms. We further discuss the consequences of this novel finding for: a) our
370 understanding of how populations under strong sexual conflict respond to global warming, b)
371 how the effects of seasonal temperature fluctuations on sexual selection may contribute to
372 balancing selection, adaptive tracking, and ultimately aid in the maintenance of standing
373 genetic variation of secondary sexual traits (i.e. lek paradox), and c) how local adaptation of
374 male harm in response to thermal ecology may foster diversification and reproductive
375 barriers between populations.

376

377 First, we show that there is quick evolution of male harm (i.e. its net impact on female
378 fitness) to temperature after 29 generations of experimental evolution under different thermal
379 regimes. Overall, we found higher levels of male harm to females at those temperatures at
380 which flies from different lines had been evolving. Male harm was lowest at 28°C in flies
381 evolved in the cold regime (20±4°C) and at 20°C in those evolved in the hot regime (28±4°C;
382 Fig. 1). In addition, flies evolved in a moderate regime (24±4°C) exhibited similar levels of
383 harm at 20, 24 and 28°C despite the fact that flies from the original founding wild population
384 exhibit substantially higher levels of harm at 24 than at 20 or 28°C (Londoño-Nieto et al.,
385 2023). In short, we found evidence that males across replicates/lines evolved in parallel to be

386 more harmful to females at their evolved thermal environment, as expected under adaptation
387 given that strong sexual selection in males has led to the evolution of male harm in this
388 species (Holland & Rice, 1999; Kawecki & Ebert, 2004; Rice, 1996).

389

390 Second, we report strong evidence of fast divergent evolution of male harm
391 mechanisms in response to cold vs. warm temperatures. Male harassment of females (pre-
392 copulatory harm) evolved to be considerably less intense and thermally plastic in lines
393 adapted to cold temperatures (16-24°C). In contrast, seminal fluid proteins (SFPs),
394 responsible for copulatory harm (Chapman et al., 1995, 2003; Wigby & Chapman, 2005) in
395 this species, characterized the evolution of male seminal proteomes at warm (24-32°C) vs.
396 cold or moderate temperatures (20-28°C) (Figs. 2-4). This finding strongly suggests that
397 temperature is likely to be a determining factor in the diversification of male harm
398 mechanisms in *Drosophila* and, potentially, other ectotherms. The evolution of decreased
399 male harassment at cold temperatures could be explained, at least partly, by natural selection
400 acting on metabolic rates, with downstream sex-specific effects on sexual selection processes
401 (Arnqvist et al., 2022). Recent theoretical and empirical developments place metabolism as a
402 causative nexus in the evolutionary interplay between ecology, life history, and sexual
403 selection (Arnqvist et al., 2022; Burger et al., 2019; Carazo, 2022). Metabolic rate is
404 intimately bound to temperature across the tree of life, but the reliance of metabolism and
405 activity on environmental temperature is particularly direct for ectotherms (Brown et al.,
406 2004). Thus, cold temperatures may place a general constraint and/or simply increase the
407 costs of male activity, consequently affecting harassment of females in ectotherms, such that
408 both evolutionary and plastic responses to cold may generally shift male-male competition
409 towards the post-copulatory arena. In accordance with this idea, the evolution of substantially
410 lower levels of harassment to females in cold experimental evolution lines parallels the

411 plastic reduction of harassment in response to cold temperature observed in the ancestral
412 population (Londoño-Nieto et al., 2023).

413 In contrast, there is ample evidence that hot temperatures have particularly strong
414 effects on proteins and sperm phenotype/function across animals (Berger et al., 2021;
415 Dougherty et al., 2024; Reinhardt et al., 2015; Sales et al., 2018; Wang & Gunderson, 2022).
416 For example, high temperatures lead to a reduction in sperm production, motility, viability
417 and longevity, consequently affecting reproductive outcomes (Wang & Gunderson, 2022).
418 Moreover, although scarce and indirect, recent findings suggest that some of these effects
419 may be mediated by temperature effects on seminal fluid proteins (Canal Domenech &
420 Fricke, 2022; Martinet et al., 2023). In particular, high temperatures increase entropy,
421 affecting protein folding and reducing the fraction of functional proteins (Berger et al., 2021).
422 This seems to suggest that hot temperatures may be particularly constraining for post-
423 copulatory sexual selection. Indeed, our results show that temperature does affect both plastic
424 and evolutionary responses of SFPs in *Drosophila*. Here, we found that SFPs responded
425 differentially to evolution at hot temperatures and, in a recent study with flies from the same
426 ancestral population, we show that hot temperature (28°C) compromises SFPs effects on
427 female receptivity, an important component of male copulatory harm to females (Londoño-
428 Nieto et al., 2023). This suggests that plastic SFP responses to hot temperature are
429 maladaptive in the ancestral wild population, and that SFPs of flies evolved at hot
430 temperatures seem to evolve quickly to recover the original levels of male harm to females.
431 To conclude, our results show that evolutionary responses to coarse-grained but natural
432 temperature fluctuations can drive the divergent evolution of male harm mechanisms. We
433 suggest that these responses may be widespread across the tree of life, potentially explaining
434 the diversity of male harm adaptations across taxa and fostering speciation by contributing to
435 establish reproductive barriers among populations.

436 Third, quick divergent evolution of pre- and copulatory mechanisms of harm would
437 only be possible via strong selection operating on high levels of standing genetic variation in
438 the ancestral population (Anderson, 2012; Barrett & Schluter, 2008). One possibility is that
439 such high levels of standing genetic variation on male secondary sexual traits are maintained
440 in the ancestral population via adaptive phenotypic plasticity (West-Eberhard, 2003). This is
441 consistent with the recent finding, in *Drosophila* from this wild population, of high levels of
442 thermal plasticity in both pre- and copulatory harm traits within the same range of
443 temperatures studied here (Londoño-Nieto et al., 2023). As stated above, male flies in the
444 ancestral wild population respond to cold temperature by decreasing harassment to females,
445 and flies evolved under the cold temperature regime evolved to harass females less and their
446 harassment was less plastic in response to temperature variation. The evolution of lower
447 harassment and the clear loss of ancestral plasticity in flies that evolved at the cold regime is
448 in fact suggestive of adaptive phenotypic plasticity in the ancestral wild population. Thus,
449 constant (and predictable) temperature fluctuations at a fine-grained ecological scale (e.g.
450 circadian variation) may, via temperature effects on sexual selection in males, contribute to
451 maintain high levels of thermal adaptive phenotypic plasticity in secondary sexual traits.
452 Such plasticity could, in turn, allow for substantial levels of cryptic genetic variation on
453 which later directional selection could operate (e.g. via selective sweeps and/or genetic
454 assimilation), which could explain the evolutionary responses in our experimental
455 populations. However, as discussed above plastic SFPs responses to hot temperatures in the
456 ancestral population appeared maladaptive (Londoño-Nieto et al., 2023). Furthermore, here
457 we report clear evidence of strong GxE interactions in thermal reaction norms for the
458 reproductive success of male genotypes derived from our ancestral wild population,
459 estimated under strong sexual selection, that were mostly characterized by clear quadratic

460 reaction norms of opposing sign (Fig. 5). This suggests the existence of fitness trade-offs and,
461 potentially, the operation of some sort of balancing selection in the ancestral population.

462 There is piling evidence for seasonal balancing selection in *Drosophila* in traits under
463 natural selection, mostly driven by adaptation to starvation, temperature stress and the
464 seasonal boom-and-burst population dynamics typical of this and other invertebrate species
465 (Bergland et al., 2014; Boulétreau-Merle et al., 1992; Hoffmann et al., 2005; Machado et al.,
466 2021; Rudman et al., 2022; Schmidt & Conde, 2006). Our results open the possibility of
467 similar balancing selection via sexual selection processes, which could contribute to explain
468 the maintenance of high levels of additive genetic variation on male secondary sexual traits, a
469 classic conundrum in evolutionary biology (i.e. the “lek paradox”; Kirkpatrick & Ryan,
470 1991). Thus, balancing selection in males may be at least partly characterised by trade-offs
471 that involve sexual selection processes, such as for example investment in pre- vs. post-
472 copulatory competition in cold vs. hot temperatures. An arising prediction of this idea is that
473 we would expect sexual differences in the type of trade-offs that result from balancing
474 selection in the wild. In accordance, temperature clines have led to a negative association
475 between resistance to starvation and cold resistance in female, but not male, *Drosophila*
476 *melanogaster* (Hoffmann et al., 2002, 2005). We suggest future studies should investigate the
477 role that temperature effects on sexual selection may play in sex-specific balancing selection,
478 and the resulting maintenance of additive genetic variation in male secondary sexual traits.

479 **Conclusions**

480 Our results show that temperature may be an important abiotic ecological factor in the
481 evolution of male harm, with implications for research on adaptation to global warming, the
482 maintenance of variability in secondary sexual traits and the diversification of male harm
483 mechanisms across populations. In addition, the finding that the male seminal proteome

484 evolves rapidly in response to temperature, and that this response is characterized by
485 differential evolution of SFPs at hot temperatures, may have implications for the study of
486 temperature effects on fertility (e.g. thermal fertility limits). We suggest future research
487 should further study plastic and evolutionary responses of SFPs to temperature and ensuing
488 effects on female reproduction and fertility at large. Finally, here we used an experimental
489 evolution approach that largely arrests the evolution of female resistance to male harm, but a
490 priority for future research should be to understand whether and how temperature may affect
491 the evolution of female resistance to harm.

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References

- Anderson, C. J. R. (2012). The Role of Standing Genetic Variation in Adaptation of Digital Organisms to a New Environment. *Artificial Life* 13, 3–10. <https://doi.org/10.7551/978-0-262-31050-5-ch001>
- Arbuthnott, D., Dutton, E. M., Agrawal, A. F., & Rundle, H. D. (2014). The ecology of sexual conflict: Ecologically dependent parallel evolution of male harm and female resistance in *Drosophila melanogaster*. *Ecol Lett*, 17(2), 221–228. <https://doi.org/10.1111/ele.12222>
- Arnqvist, G. (2020). Mixed Models Offer No Freedom from Degrees of Freedom. *Trends in Ecology & Evolution*, 35(4), 329–335. <https://doi.org/10.1016/j.tree.2019.12.004>
- Arnqvist, G., Rönn, J., Watson, C., Goenaga, J., & Immonen, E. (2022). Concerted evolution of metabolic rate, economics of mating, ecology and pace-of-life across seed beetles. *PNAS*.
- Arnqvist, G., & Rowe, C. (2005). *Sexual Conflict*. Princeton University Press.
- Barrett, R., & Schluter, D. (2008). Adaptation from standing genetic variation. *Trends in Ecology & Evolution*, 23(1), 38–44. <https://doi.org/10.1016/j.tree.2007.09.008>
- Bastock, M., & Manning, A. (1955). The courtship behaviour of *Drosophila melanogaster*. *Behaviour*, 8, 85–111.
- Bates, D., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1). <https://doi.org/10.18637/jss.v067.i01>
- Berger, D., Stångberg, J., Baur, J., & Walters, R. (2021). Elevated temperature increases genome-wide selection on de novo mutations. *Proceedings of the Royal Society B: Biological Sciences*, 287. <https://doi.org/10.1098/rspb.2020.3094>
- Bergland, A. O., Behrman, E. L., O'Brien, K. R., Schmidt, P. S., & Petrov, D. A. (2014). Genomic Evidence of Rapid and Stable Adaptive Oscillations over Seasonal Time Scales in *Drosophila*. *PLoS Genetics*, 10(11), e1004775. <https://doi.org/10.1371/journal.pgen.1004775>
- Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H., & White, J.-S. S. (2009). Generalized linear mixed models: A practical guide for ecology and evolution. *Trends in Ecology & Evolution*, 24(3), 127–135. <https://doi.org/10.1016/j.tree.2008.10.008>

- Bonduriansky, R., Maklakov, A., Zajitschek, F., & Brooks, R. (2008). Sexual selection, sexual conflict and the evolution of ageing and life span. *Functional Ecology*, 22(3), 443–453. <https://doi.org/10.1111/j.1365-2435.2008.01417.x>
- Boulétreau-Merle, J., Fouillet, P., & Terrier, O. (1992). Clinal and seasonal variations in initial retention capacity of virgin *Drosophila melanogaster* females as a strategy for fitness. *Evolutionary Ecology*, 6(3), 223–242. <https://doi.org/10.1007/BF02214163>
- Bretman, A., & Fricke, C. (2019). Exposure to males, but not receipt of sex peptide, accelerates functional ageing in female fruit flies. *Functional Ecology*, 33(8), 1459–1468. <https://doi.org/10.1111/1365-2435.13339>
- Brooks, M. E., Kristensen, K., Benthem, K. J., van, Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Mächler, M., & Bolker, B. M. (2017). glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal*, 9(2), 378. <https://doi.org/10.32614/RJ-2017-066>
- Brown, J. H., Gillooly, J. F., Allen, A. P., Savage, V. M., & West, G. B. (2004). TOWARD A METABOLIC THEORY OF ECOLOGY. *Ecology*, 85(7), 1771–1789. <https://doi.org/10.1890/03-9000>
- Burger, J. R., Hou, C., & Brown, J. H. (2019). Toward a metabolic theory of life history. *Proceedings of the National Academy of Sciences*, 116(52), 26653–26661. <https://doi.org/10.1073/pnas.1907702116>
- Cally, J. G., Stuart-Fox, D., & Holman, L. (2019). Meta-analytic evidence that sexual selection improves population fitness. *Nat Commun*, 10(1), 2017. <https://doi.org/10.1038/s41467-019-10074-7>
- Canal Domenech, B., & Fricke, C. (2022). Recovery from heat-induced infertility—A study of reproductive tissue responses and fitness consequences in male *Drosophila melanogaster*. *Ecology and Evolution*, 12(12), e9563. <https://doi.org/10.1002/ece3.9563>
- Carazo, P. (2022). Metabolism as a screenwriter in the female–male coevolutionary play. *Proceedings of the National Academy of Sciences*, 119(39), e2213208119. <https://doi.org/10.1073/pnas.2213208119>

- Carazo, P., Tan, C. K. W., Allen, F., Wigby, S., & Pizzari, T. (2014). Within-group male relatedness reduces harm to females in *Drosophila*. *Nature*, *505*(7485), 672–675.
<https://doi.org/10.1038/nature12949>
- Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M. F., Smith, H. K., & Partridge, L. (2003). The sex peptide of *Drosophila melanogaster*: Female post-mating responses analyzed by using RNA interference. *Proc Natl Acad Sci USA*, *100*.
<https://doi.org/10.1073/pnas.1631635100>
- Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F., & Partridge, L. (1995). Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*, *373*(6511), 241.
- Clancy, D. J., & Kennington, J. (2001). A simple method to achieve consistent larval density in bottle cultures. *Drosophila Information Service*, *84*(January), 168–169.
- Connolly, K., & Cook, R. (1973). Rejection Responses By Female *Drosophila Melanogaster*: Their Ontogeny, Causality and Effects Upon the Behaviour of the Courting Male. *Behaviour*, *44*(1–2), 142–165. <https://doi.org/10.1163/156853973x00364>
- Crudginton, H. S., & Siva-Jothy, M. T. (2000). Genital damage, kicking and early death. *Nature*, *407*(6806), 855–856. <https://doi.org/10.1038/35038154>
- Dougherty, L. R., Frost, F., Maenpaa, M. I., Rowe, M., Cole, B. J., Vasudeva, R., Pottier, P., Schultner, E., Macartney, E. L., Lindenbaum, I., Smith, J. L., Carazo, P., Graziano, M., Weaving, H., Domenech, B. C., Berger, D., Meena, A., Bishop, T. R., Noble, D. W. A., ... Price, T. A. R. (2024). A systematic map of studies testing the relationship between temperature and animal reproduction. *Ecological Solutions and Evidence*, *5*.
- Dukas, R. (2020). Natural history of social and sexual behavior in fruit flies. *Scientific Reports*, *10*(1), 21932. <https://doi.org/10.1038/s41598-020-79075-7>
- Falconer, D. (1996). *Introduction to quantitative genetics* (Fourth edition).
- Filice, D. C. S., Bhargava, R., & Dukas, R. (2020). Plasticity in male mating behavior modulates female life history in fruit flies. *Evolution*, *74*(2), 365–376. <https://doi.org/10.1111/evo.13926>

- Flintham, E. O., Savolainen, V., & Mullon, C. (2023). Male harm offsets the demographic benefits of good genes. *Proceedings of the National Academy of Sciences*, *120*(10), e2211668120.
<https://doi.org/10.1073/pnas.2211668120>
- Friedman, J., Hastie, T., & Tibshirani, R. (2010). Regularization Paths for Generalized Linear Models via Coordinate Descent. *Journal of Statistical Software*, *33*(1).
<https://doi.org/10.18637/jss.v033.i01>
- Fromonteil, S., Marie-Orleach, L., Winkler, L., & Janicke, T. (2023). Sexual selection in females and the evolution of polyandry. *PLOS Biology*, *21*(1), e3001916.
<https://doi.org/10.1371/journal.pbio.3001916>
- Gao, C. H., Yu, G., & Cai, P. (2021). ggVennDiagram: An Intuitive, Easy-to-Use, and Highly Customizable R Package to Generate Venn Diagram. *Frontiers in Genetics*, *12*(September).
<https://doi.org/10.3389/fgene.2021.706907>
- García-Roa, R., Chirinos, V., & Carazo, P. (2019). The ecology of sexual conflict: Temperature variation in the social environment can drastically modulate male harm to females. *Functional Ecology*, *33*(4), 681–692. <https://doi.org/10.1111/1365-2435.13275>
- García-Roa, R., Garcia-Gonzalez, F., Noble, D. W. A., & Carazo, P. (2020). Temperature as a modulator of sexual selection. *Biological Reviews*, *95*(6), 1607–1629.
<https://doi.org/10.1111/brv.12632>
- Gaujoux, R., & Seoighe, C. (2010). A flexible R package for nonnegative matrix factorization. *BMC Bioinformatics*, *11*. <https://doi.org/10.1186/1471-2105-11-367>
- Gavrilets, S. (2014). Is Sexual Conflict an ‘Engine of Speciation’? *Cold Spring Harbor Perspectives in Biology*, *6*(12). <http://cshperspectives.cshlp.org/content/6/12/a017723.abstract>
- Gillet, L. C., Navarro, P., Tate, S., Röst, H., Selevsek, N., Reiter, L., Bonner, R., & Aebersold, R. (2012). Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: A new concept for consistent and accurate proteome analysis. *Molecular and Cellular Proteomics*, *11*(6), 1–17. <https://doi.org/10.1074/mcp.O111.016717>

- Gómez-Llano, M., Faria, G. S., García-Roa, R., Noble, D. W. A., & Carazo, P. (2024). Male harm suppresses female fitness, affecting the dynamics of adaptation and evolutionary rescue. *Evolution Letters*, 8, 149–160.
- Hoffmann, A. A., Anderson, A., & Hallas, R. (2002). Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecology Letters*, 5(5), 614–618.
<https://doi.org/10.1046/j.1461-0248.2002.00367.x>
- Hoffmann, A. A., Hallas, R., Anderson, A. R., & Telonis-Scott, M. (2005). Evidence for a robust sex-specific trade-off between cold resistance and starvation resistance in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, 18(4), 804–810.
<https://doi.org/10.1111/j.1420-9101.2004.00871.x>
- Holland, B., & Rice, W. R. (1999). Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *PNAS*, 96, 5083–5088.
- Hopkins, B. R., Sepil, I., Bonham, S., Miller, T., Charles, P. D., Fischer, R., Kessler, B. M., Wilson, C., & Wigby, S. (2019a). BMP signaling inhibition in *Drosophila* secondary cells remodels the seminal proteome and self and rival ejaculate functions. *Proceedings of the National Academy of Sciences*, 116(49), 24719–24728. <https://doi.org/10.1073/pnas.1914491116>
- Hopkins, B. R., Sepil, I., Thézénas, M.-L., Craig, J. F., Miller, T., Charles, P. D., Fischer, R., Kessler, B. M., Bretman, A., Pizzari, T., & Wigby, S. (2019b). Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 116(36), 17925–17933.
<https://doi.org/10.1073/pnas.1906149116>
- Janicke, T. A.-O., Haderer, I. K., Lajeunesse, M. J., & Anthes, N. (2016). Darwinian sex roles confirmed across the animal kingdom. *Science Advances*, 2, e1500983.
- Kassambara, A., & Kosinski, M. (2018). *Survminer: Drawing Survival Curves using 'ggplot2'*. R package version 0.4.3. <https://CRAN.R-project.org/package=survminer>
- Kawecki, T. J., & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology Letters*, 7(12), 1225–1241. <https://doi.org/10.1111/j.1461-0248.2004.00684.x>

- Kirkpatrick, M., & Ryan, M. J. (1991). The evolution of mating preferences and the paradox of the lek. *Nature*, *350*, 33–38.
- Koene, J. M., & Schulenburg, H. (2005). Shooting darts: Co-evolution and counter-adaptation in hermaphroditic snails. *BMC Evolutionary Biology*, *5*(1), 25–38. <https://doi.org/10.1186/1471-2148-5-25>
- Konishi, S., & Kitagawa, G. (2008). Information Criterion. In *Information Criteria and Statistical Modeling* (pp. 29–74). Springer New York.
- LaFlamme, B. A., Avila, F. W., Michalski, K., & Wolfner, M. F. (2014). A *Drosophila* Protease Cascade Member, Seminal Metalloprotease-1, Is Activated Stepwise by Male Factors and Requires Female Factors for Full Activity. *Genetics*, *196*(4), 1117–1129. <https://doi.org/10.1534/genetics.113.160101>
- Lange, R., Reinhardt, K., Michiels, N. K., & Anthes, N. (2013). Functions, diversity, and evolution of traumatic mating: Function and evolution of traumatic mating. *Biological Reviews*, *88*(3), 585–601. <https://doi.org/10.1111/brv.12018>
- Le Galliard, J. F., Fitze, P. S., Ferrière, R., & Clobert, J. (2005). Sex ratio bias, male aggression, and population collapse in lizards. *Proc.Natl.Acad.Sci.USA*, *102*(50), 18231–18236.
- Londoño-Nieto, C., García-Roa, R., Garcia-Co, C., González, P., & Carazo, P. (2023). Thermal phenotypic plasticity of pre- and post-copulatory male harm buffers sexual conflict in wild *Drosophila melanogaster*. *eLife*, *12*, e84759. <https://doi.org/10.7554/eLife.84759>
- Lorch, P. D., Proulx, S., Rowe, L., & Day, T. (2003). Condition-dependent sexual selection can accelerate adaptation. *Evolutionary Ecology Research*, *5*, 867–881.
- Lumley, A. J., Michalczyk, L., Kitson, J. J., Spurgin, L. G., Morrison, C. A., Godwin, J. L., Dickinson, M. E., Martin, O. Y., Emerson, B. C., Chapman, T., & Gage, M. J. (2015). Sexual selection protects against extinction. *Nature*. <https://doi.org/10.1038/nature14419>
- Machado, H. E., Bergland, A. O., Taylor, R., Tilk, S., Behrman, E., Dyer, K., Fabian, D. K., Flatt, T., González, J., Karasov, T. L., Kim, B., Kozeretska, I., Lazzaro, B. P., Merritt, T. J., Pool, J. E., O'Brien, K., Rajpurohit, S., Roy, P. R., Schaeffer, S. W., ... Petrov, D. A. (2021). Broad

- geographic sampling reveals the shared basis and environmental correlates of seasonal adaptation in *Drosophila*. *eLife*, *10*, e67577. <https://doi.org/10.7554/eLife.67577>
- Martinet, B., Przybyla, K., Decroo, C., Wattiez, R., & Aron, S. (2023). Proteomic differences in seminal fluid of social insects whose sperm differ in heat tolerance. *Royal Society Open Science*, *10*(11), 231389. <https://doi.org/10.1098/rsos.231389>
- Nakagawa, S., & Schielzeth, H. (2013). A general and simple method for obtaining R² from generalized linear mixed-effects models. *Methods in Ecology and Evolution*, *4*(2), 133–142.
- Parker, G. (1979). Sexual selection and sexual conflict. *Sexual Selection and Reproductive Competition in Insects*, *123*, 166.
- Partridge, L., & Fowler, K. (1990). Non-mating costs of exposure to males in female *Drosophila melanogaster*. *Journal of Insect Physiology*, *36*(6), 419–425. [https://doi.org/10.1016/0022-1910\(90\)90059-O](https://doi.org/10.1016/0022-1910(90)90059-O)
- Perry, J. C., Garroway, C. J., & Rowe, L. (2017). The role of ecology, neutral processes and antagonistic coevolution in an apparent sexual arms race. *Ecol Lett*, *20*(9), 1107–1117. <https://doi.org/10.1111/ele.12806>
- Perry, J. C., & Rowe, L. (2018). Sexual conflict in its ecological setting. *Philos Trans R Soc Lond B Biol Sci*, *373*(1757). <https://doi.org/10.1098/rstb.2017.0418>
- Pitnick, S., & Garcia-Gonzalez, F. (2002). Harm to females increases with male body size in *Drosophila melanogaster*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *269*(1502), 1821. <https://doi.org/10.1098/rspb.2002.2090>
- Pizzari, T., & Snook, R. R. (2003). Perspective: Sexual Conflict and Sexual Selection: Chasing Away Paradigm Shifts. *Evolution*, *57*(6), 1223–1236. <https://doi.org/10.2307/3448846>
- Rankin, D. J., & Kokko, H. (2006). Sex, death and tragedy. *Trends in Ecology & Evolution*, *21*(5), 225–226. <https://doi.org/10.1016/j.tree.2006.02.013>
- Ravi Ram, K., Sirot, L. K., & Wolfner, M. F. (2006). Predicted seminal astacin-like protease is required for processing of reproductive proteins in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, *103*(49), 18674–18679. <https://doi.org/10.1073/pnas.0606228103>

- Reinhardt, K., Dobler, R., & Abbott, J. (2015). An Ecology of Sperm: Sperm Diversification by Natural Selection. *Annual Review of Ecology, Evolution, and Systematics*, *46*(1), 435–459. <https://doi.org/10.1146/annurev-ecolsys-120213-091611>
- Rice, W. R. (1996). Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*, *381*(6579), 232–234. <https://doi.org/10.1038/381232a0>
- Rice, W. R., Linder, J. E., Friberg, U., Lew, T. A., Morrow, E. H., & Stewart, A. D. (2005). Inter-locus antagonistic coevolution as an engine of speciation: Assessment with hemiclonal analysis. *Proceedings of the National Academy of Sciences*, *102*(suppl 1), 6527–6534. <https://doi.org/10.1073/pnas.0501889102>
- Rohart, F., Gautier, B., Singh, A., & Lê Cao, K.-A. (2017). mixOmics: An R package for ‘omics feature selection and multiple data integration. *PLOS Computational Biology*, *13*(11), e1005752. <https://doi.org/10.1371/journal.pcbi.1005752>
- Rowe, L., & Houle, D. (1996). The lek paradox and the capture of genetic variance by condition dependent traits. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *263*(1375), 1415. <https://doi.org/10.1098/rspb.1996.0207>
- Rudman, S. M., Greenblum, S. I., Rajpurohit, S., Betancourt, N. J., Hanna, J., Tilk, S., Yokoyama, T., Petrov, D. A., & Schmidt, P. (2022). Direct observation of adaptive tracking on ecological time scales in *Drosophila*. *Science*, *375*(6586), eabj7484. <https://doi.org/10.1126/science.abj7484>
- Sales, K., Vasudeva, R., Dickinson, M. E., Godwin, J. L., Lumley, A. J., Michalczyk, Å., Hebberecht, L., Thomas, P., Franco, A., & Gage, M. J. G. (2018). Experimental heatwaves compromise sperm function and cause transgenerational damage in a model insect. *Nature Communications*, *9*(1), 4771. <https://doi.org/10.1038/s41467-018-07273-z>
- Schmidt, P. S., & Conde, D. R. (2006). ENVIRONMENTAL HETEROGENEITY AND THE MAINTENANCE OF GENETIC VARIATION FOR REPRODUCTIVE DIAPAUSE IN *DROSOPHILA MELANOGASTER*. *Evolution*, *60*(8), 1602–1611. <https://doi.org/10.1111/j.0014-3820.2006.tb00505.x>

- Sepil, I., Hopkins, B. R., Dean, R., Thézénas, M.-L., Charles, P. D., Konietzny, R., Fischer, R., Kessler, B. M., & Wigby, S. (2019). Quantitative Proteomics Identification of Seminal Fluid Proteins in Male *Drosophila melanogaster*. *Molecular & Cellular Proteomics*, *18*, S46–S58. <https://doi.org/10.1074/mcp.RA118.000831>
- Sirot, L. K., Wolfner, M. F., & Wigby, S. (2011). Protein-specific manipulation of ejaculate composition in response to female mating status in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*, *108*(24), 9922–9926. <https://doi.org/10.1073/pnas.1100905108>
- Smithson, M., & Verkuilen, J. (2006). A better lemon squeezer? Maximum-likelihood regression with beta-distributed dependent variables. *Psychological Methods*, *11*(1), 54–71. <https://doi.org/10.1037/1082-989X.11.1.54>
- Teseo, S., Veerus, L., Moreno, C., & Mery, F. (2016). Sexual harassment induces a temporary fitness cost but does not constrain the acquisition of environmental information in fruit flies. *Biology Letters*, *12*(1), 20150917. <https://doi.org/10.1098/rsbl.2015.0917>
- Therneau, T. (2022). *Coxme: Mixed Effects Cox Models. R package version 2.2-1*. 1–21.
- Wang, W. W.-Y., & Gunderson, A. R. (2022). The Physiological and Evolutionary Ecology of Sperm Thermal Performance. *Frontiers in Physiology*, *13*, 754830. <https://doi.org/10.3389/fphys.2022.754830>
- West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution*. Oxford University Press.
- Wigby, S., & Chapman, T. (2005). Sex peptide causes mating costs in female *Drosophila melanogaster*. *Current Biology*, *15*(4), 316–321.
- Yun, L., Agrawal, A. F., & Rundle, H. D. (2021). On Male Harm: How it is Measured and How it Evolves in Different Environments. *The American Naturalist*, *81*(1), 1–37.
- Yun, L., Chen, P. J., Kwok, K. E., Angell, C. S., Rundle, H. D., & Agrawal, A. F. (2018). Competition for mates and the improvement of nonsexual fitness. *Proc Natl Acad Sci U S A*. <https://doi.org/10.1073/pnas.1805435115>
- Yun, L., Chen, P. J., Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). The physical environment mediates male harm and its effect on selection in females. *Proc Biol Sci*, *284*(1858). <https://doi.org/10.1098/rspb.2017.0424>

Zou, H., & Hastie, T. (2005). Regularization and Variable Selection Via the Elastic Net. *Journal of the Royal Statistical Society Series B: Statistical Methodology*, 67(2), 301–320.

<https://doi.org/10.1111/j.1467-9868.2005.00503.x>

Table 1. Sample sizes for female reproductive success and survivorship experiments.

| Temperature treatment | Mating system | Regime of evolution | | |
|-----------------------|---------------|---------------------|----------|-----|
| | | Cold | Moderate | Hot |
| 20° | Monogamy | 166 | 168 | 157 |
| | Polyandry | 171 | 167 | 167 |
| 24° | Monogamy | 162 | 162 | 153 |
| | Polyandry | 163 | 165 | 152 |
| 28° | Monogamy | 165 | 172 | 165 |
| | Polyandry | 163 | 173 | 169 |

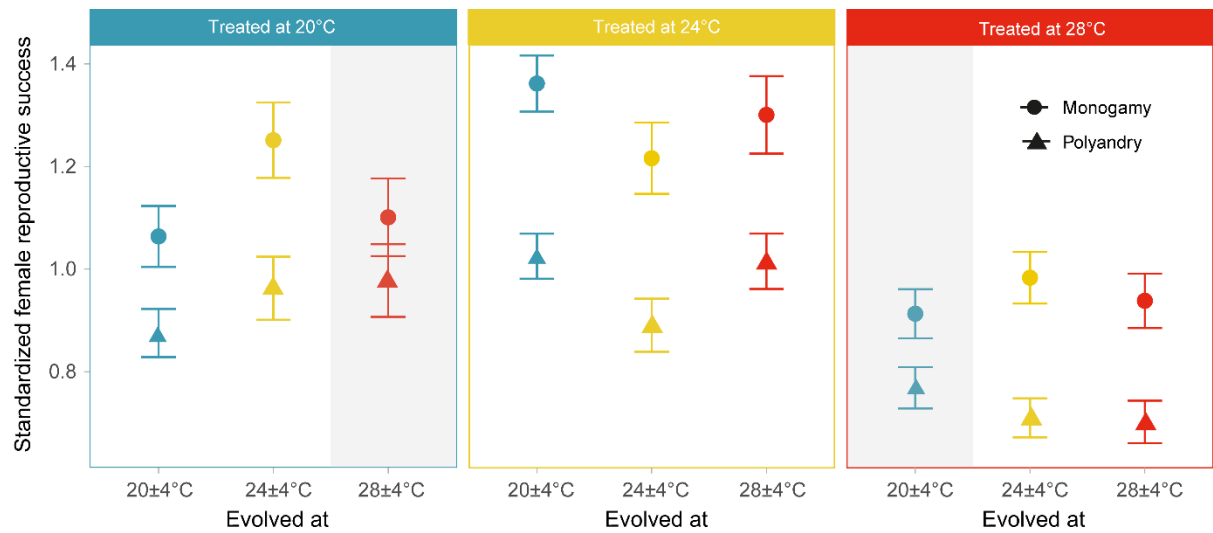


Figure 1 | Effect of mating system, temperature treatment and experimental evolution regime on female fitness. Female reproductive success (mean \pm s.e. of four replicates) across mating systems (monogamy and polyandry), temperature treatments (20,24 and 28°C) and experimental evolution thermal regimes (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 were standardized for each experimental evolution line.

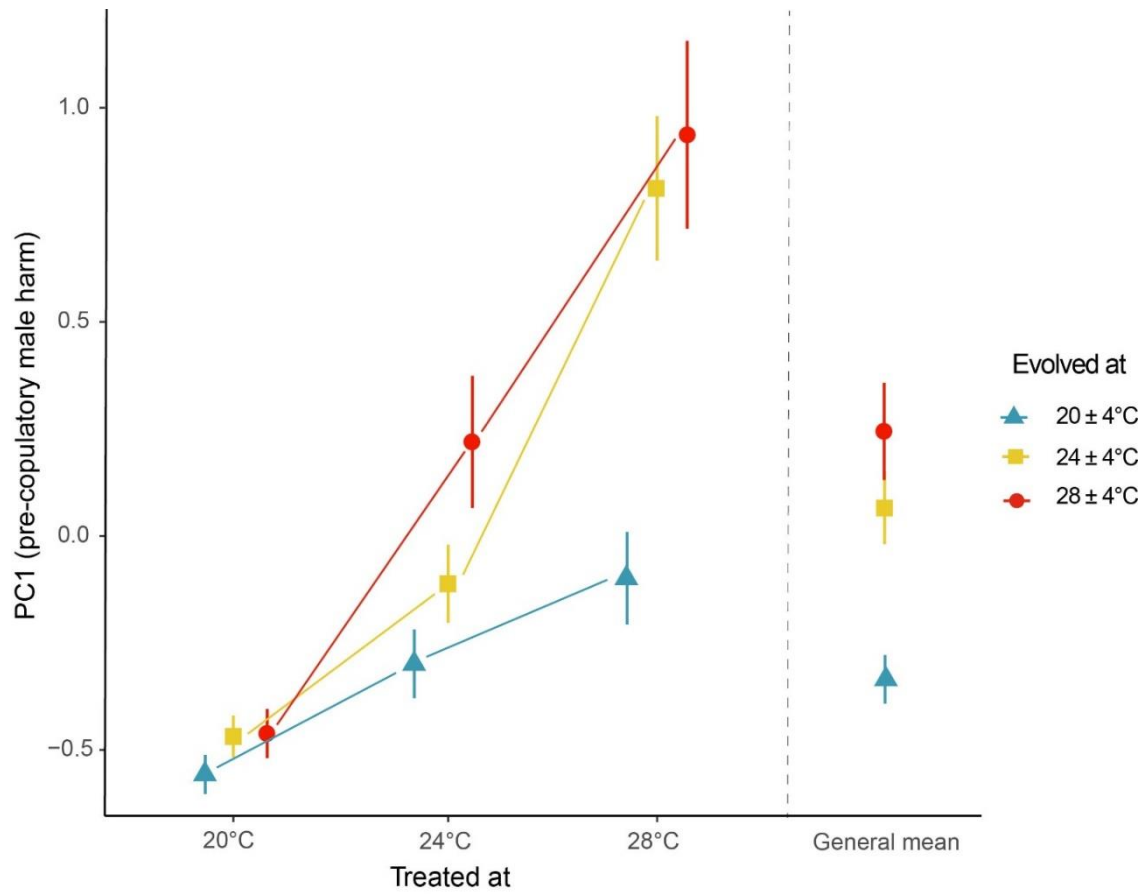


Figure 2 | Effect of temperature treatment and experimental evolution regime on pre-copulatory male harm. Frequency patterns (mean \pm s.e.) for the PC1 from a PCA in which all behaviours involved in pre-copulatory harm (courtship intensity, female rejection and male-male aggression) were examined together for increased conflict (i.e., polyandry). We took this PC1 as an overall index of male harassment to females.

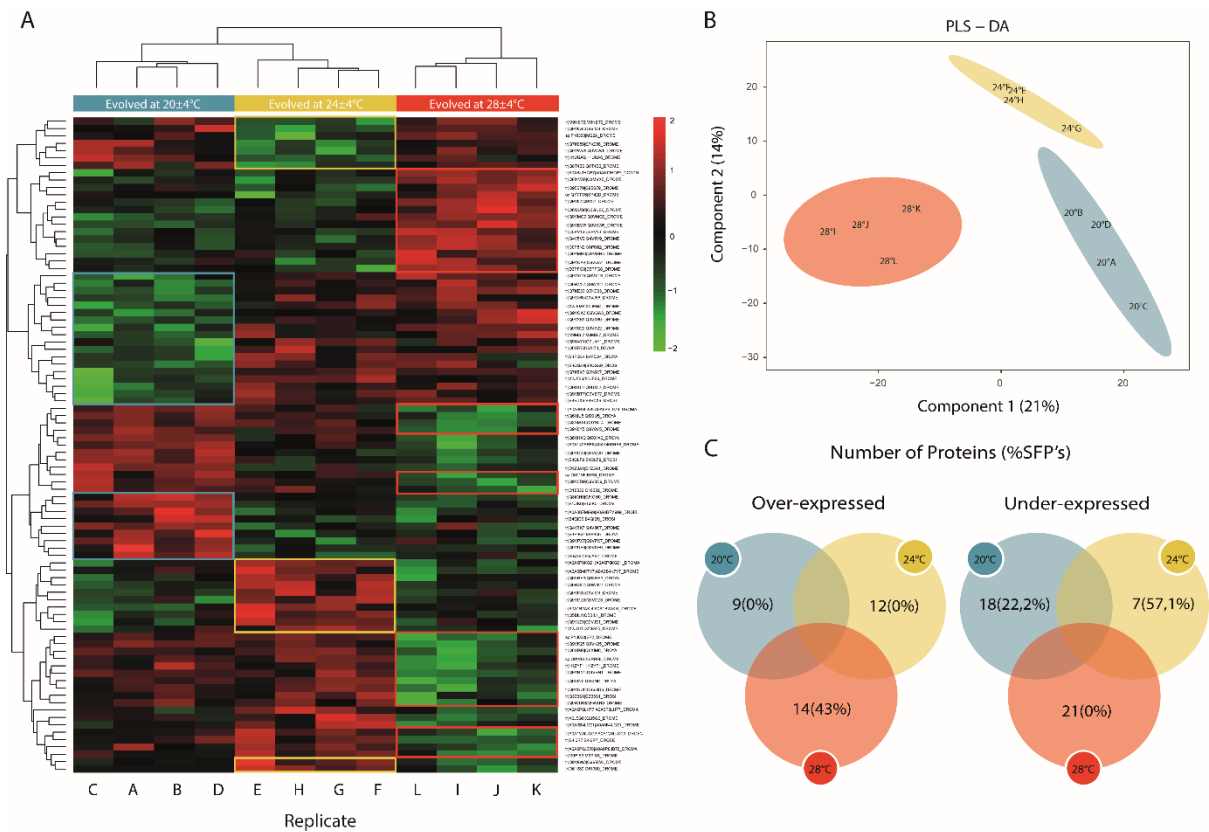


Figure 4 | Effect of experimental evolution regime on mated male's seminal proteome production. A) Heatmap showing the abundance of 89 proteins selected by the Elastic net regression. Each cell gives the across-biological replicate mean for that protein in a given experimental evolution thermal regime 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 89 proteins selected), and the corresponding seminal fluid proteins percentage, by males evolved in each experimental evolution thermal regime.

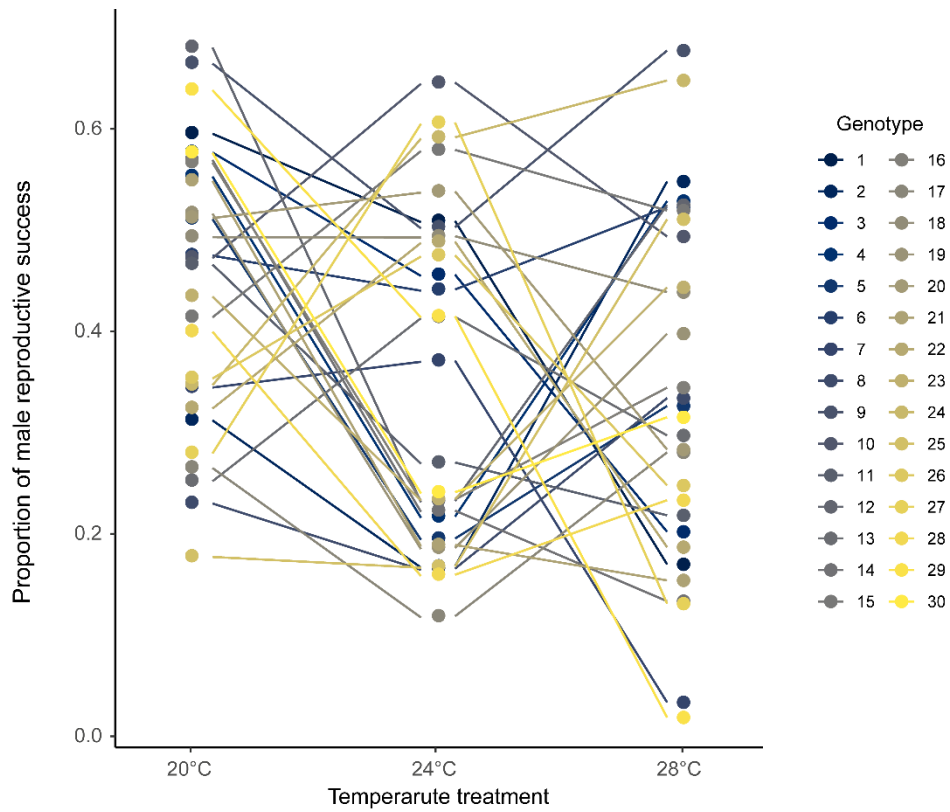


Figure 5 | Genotype-by-environment interactions for male reproductive success. Reaction norms for male reproductive success in 30 genotypes analysed across three temperature treatments.