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

Claudia Londoño-Nieto (claudia.londono@uv.es)[1], Michael Butler (miquel.butler.mb@gmail.com)[1], Roberto García-Roa (roberto.garcia.roa@gmail.com)[1,2], and Pau Carazo (pau.carazo@uv.es)[1]

[1]Ethology Lab, Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, Valencia, Spain.

[2] Department of Biology, Lund University, Lund, Sweden.

Short title: Temperature and the evolutionary diversification of sexual conflict

Keywords: Temperature, sexual conflict, experimental evolution, ecology, sexual selection, SFPs, male harm, Drosophila

Type of article: Letter. **Number of words in the abstract:** 150. **Number of words in the main text**: 5830. **Number of references**: 91. **Number of figures**: 5. **Number of tables:** 1.

Authors for correspondence: Claudia Patricia Londoño Nieto (Claudia.londono@uv.es) +37 674572407. Pau Carazo (pau.carazo@uv.es) +34 963544051.

Statement of authorship: Claudia Londoño-Nieto: Conceptualization, data collection and curation, formal analysis, investigation, methodology, writing; Roberto García-Roa: Conceptualization, supervision, investigation, methodology, data collection – supporting, writing - review; Michael Butler: Data collection and curation, methodology; Pau Carazo:

Conceptualization, resources, supervision, funding acquisition, validation, investigation, methodology, data collection - supporting, writing - review and editing.

Data accessibility statement: Data will be accessible on Dryad once the manuscript is published (https://doi.org/10.5061/dryad.7sqv9s51x).

ABSTRACT

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

Introduction

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

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

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

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

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

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

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

Methods

Experimental evolution design

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

selection for female resistance should be minimized (Bonduriansky et al., 2008; Filice et al., 2020). Populations were assayed after 29-30 generations of experimental evolution and two generations of common garden at 24±4°C to control for parental and grand-parental effects. Experimental evolution started in February 2020 for all lines, and finished in August 2021 for the hot regime, in October 2021 for the moderate regime and in April 2022 for the cold regime. Differences in evolution time are due to differences in development time at each thermal regime. Populations from the hot regime were assayed between September and October 2021, from the moderate regime between November and December 2021 and from the cold regime between June and July 2022.

Male harm and behavioural assays (experiment 1)

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

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

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

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

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

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

Proteomics assays (experiment 2)

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

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

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

GxE assay (experiment 3)

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

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

Results

Harm to females is higher at evolved temperatures

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

Cold temperatures decrease the levels and thermal plasticity of male harassment

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

Conclusions

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

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

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 virginDrosophila 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

Crudgington, 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., Ferriäre, 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

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
20º	Monogamy	166	168	157
	Polyandry	171	167	167
24º	Monogamy	162	162	153
	Polyandry	163	165	152
28º	Monogamy	165	172	165
	Polyandry	163	173	169

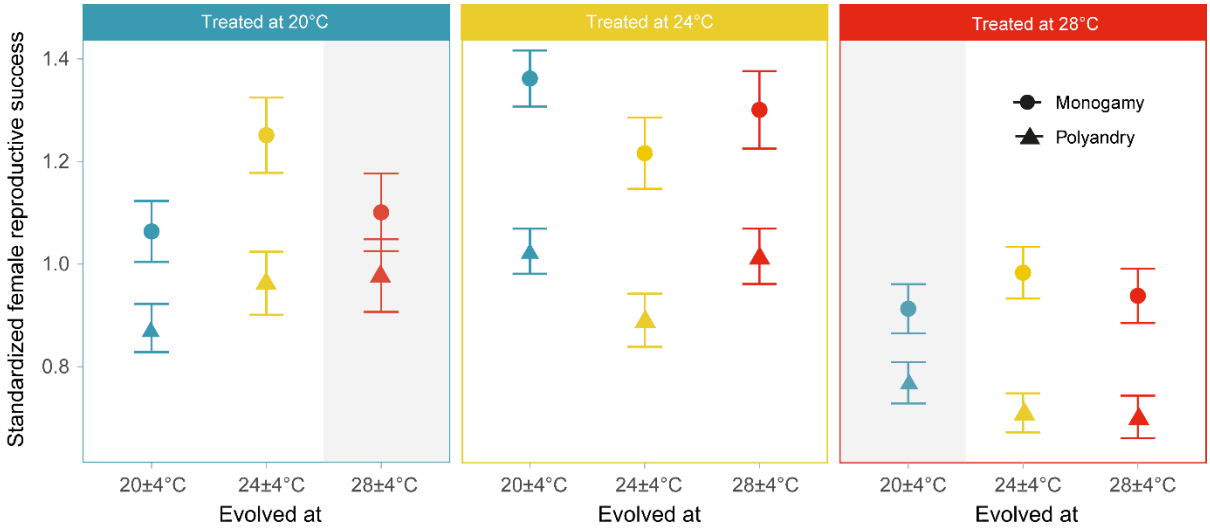

Figure 1 | Effect of mating system, temperature treatment and experimental evolution regime on female fitness. Female reproductive success (mean ± s.e. of four replicates) across mating systems (monogamy and polyandry), temperature treatments (20,24 and 28°C) and experimental evolution thermal regimes (20±4, 24±4 and 28±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.

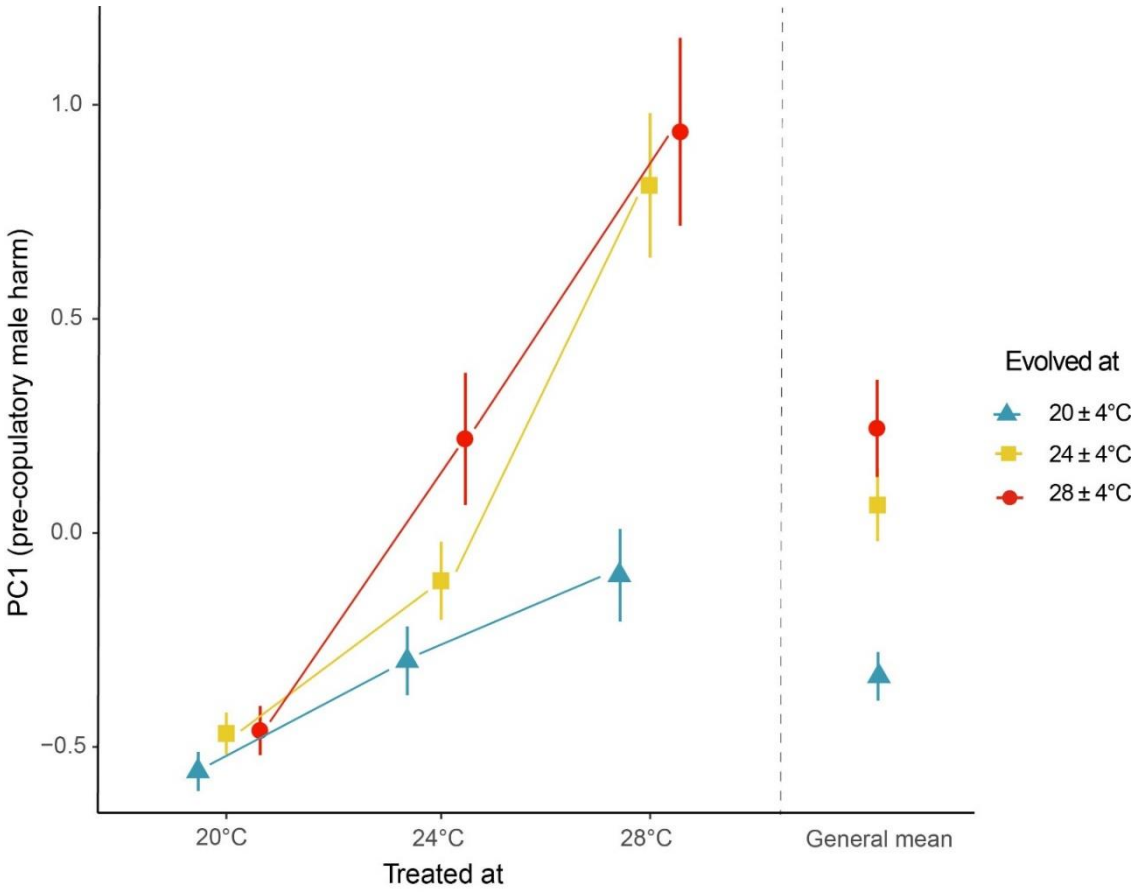

Figure 2 | **Effect of temperature treatment and experimental evolution regime on pre-copulatory male harm**. Frequency patterns (mean ± s.e.) for the PC1 from a PCA in which all behaviours involved in pre-copulatory harm (courtship intensity, female rejection and male-male aggression) were examined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

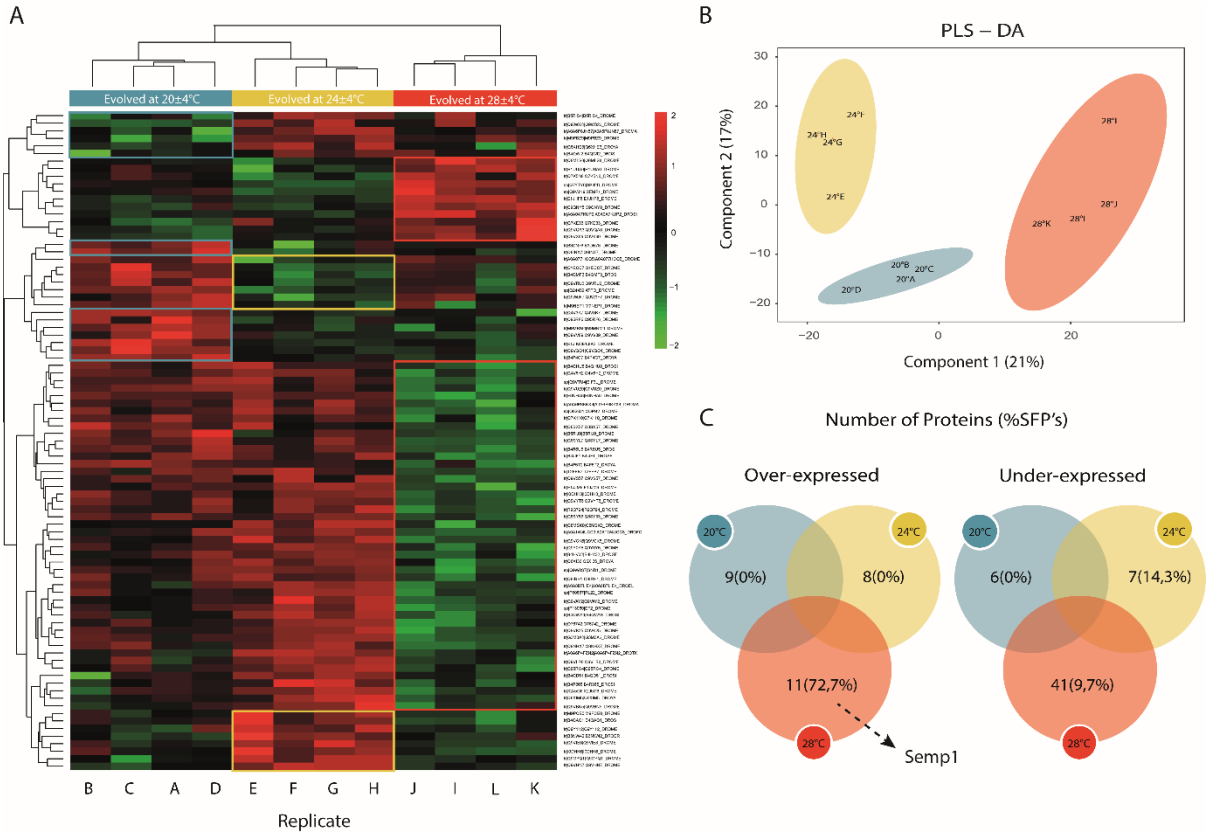

Figure 3 | Effect of experimental evolution regime on virgin male's seminal proteome production. A) Heatmap showing the abundance of 87 proteins selected by the Elastic net regression. Each cell gives the across-biological replicate mean for that protein in each experimental evolution thermal regime 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 (inside the 87 proteins selected), and the corresponding seminal fluid proteins percentage, by males evolved in each experimental evolution thermal regime. Semp1 protein (Q9VJN9) was singularly over-expressed by males evolved in hot regime and it is known as a seminal fluid metalloprotease which is transferred to females during mating and is required for processing of ovulin and sperm storage proteins (two of the best known SFP´s in *D. melanogaster*) in mated females.

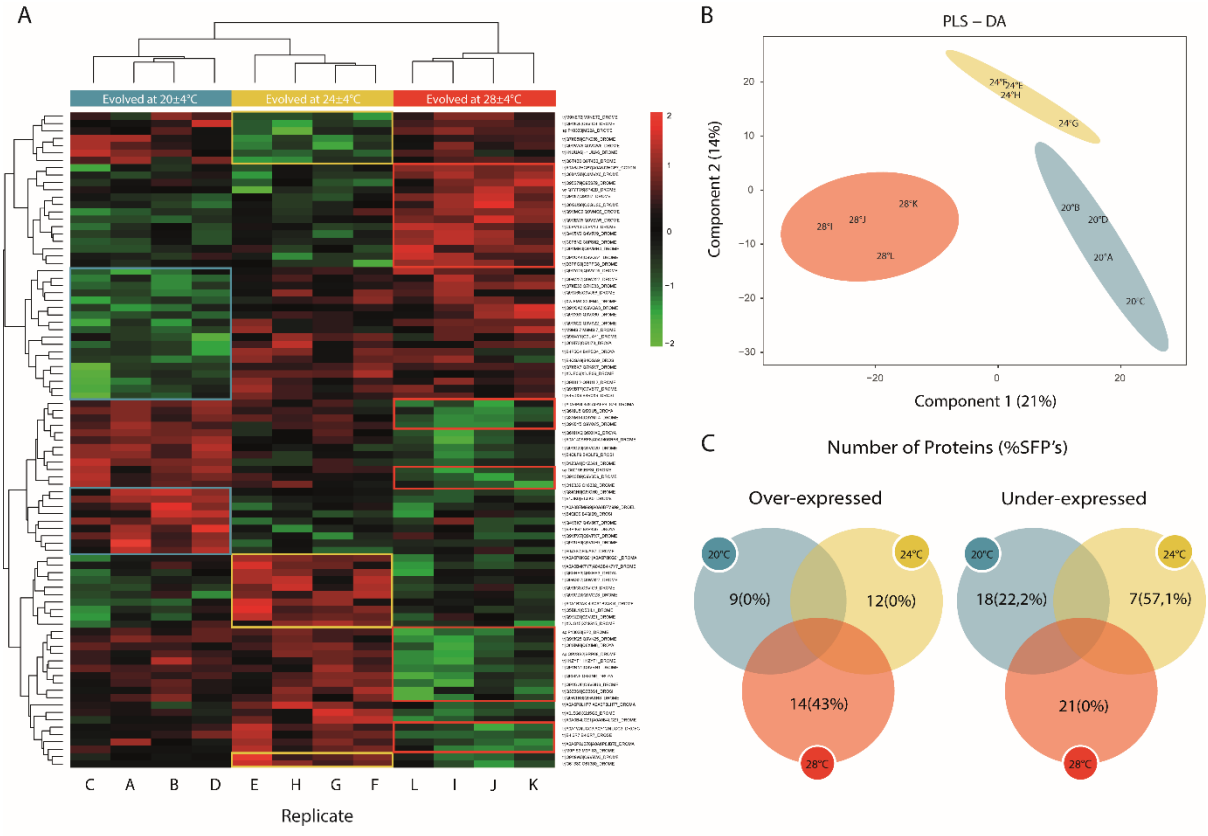

Figure 4 | Effect of experimental evolution regime on mated male's seminal proteome production. A) Heatmap showing the abundance of 89 proteins selected by the Elastic net regression. Each cell gives the across-biological replicate mean for that protein in a given experimental evolution thermal regime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

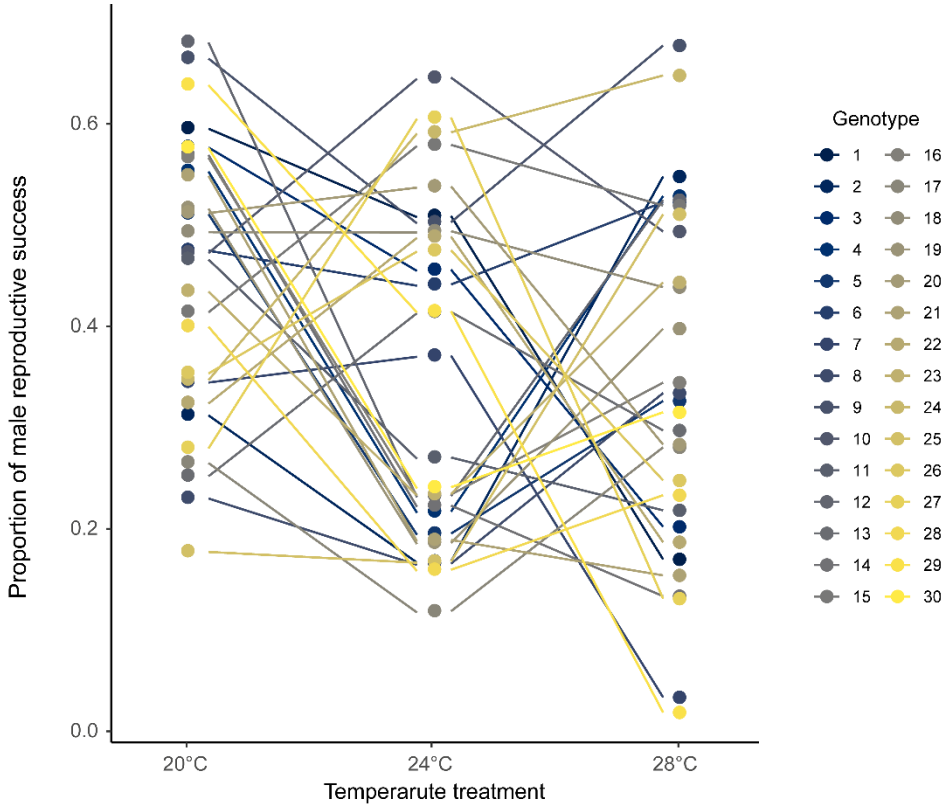

Figure 5 | Genotype-by-environment interactions for male reproductive success. Reaction norms for male reproductive success in 30 genotypes analysed across three temperature treatments.