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

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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 male harm via harassment and seminal fluid proteins (SFPs), to show that temperature drives the divergent evolution of sexual conflict. At the cold regime, evolution resulted in reduced and less plastic harassment (i.e. pre-copulatory harm) while, at the hot regime, it was characterized by responses in the seminal proteome driven by differential expression of SFPs. Our results suggest 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; Martinez-Ruiz & Robert, 2017; Martinossi-Allibert et al., 2019). 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 (Agrawal, 2001; Whitlock & Agrawal, 2009). However, the same divergent selective pressures that make sexual selection such an effective evolutionary sieve also set the scene for sexual conflict, 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). 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).

Harmful male adaptations to females (male harm) are pervasive, diverse and sophisticated across the tree of life. 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 a variety of male harm adaptations that are similarly widespread, ranging from potentially harmful ejaculates (Wigby & Chapman, 2005) to adaptations for traumatic insemination (Crudgington & Siva-Jothy, 2000). 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 can impact 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 (Flintham et al., 2023; Gómez-Llano et al., 2024; Pitnick & García-González, 2002). 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, is also a recipe for intense sexual conflict. Understanding what factors determine whether strong sexual selection and resulting conflict leads to harm to females, and the nature and diversity of its underlying traits, is a central question in evolutionary biology.

A growing body of research highlights ecology as a crucial factor for understanding 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 the advantages of good-genes selection (Londoño-Nieto et al., 2023; Yun et al., 2017,2018). Recent studies show that environmental factors such as spatial complexity (Berger & Liljestrand-Rönn, 2024; MacPherson et al., 2018; Malek & Long, 2019), nutritional status (Fricke et al., 2010), or sex ratio and population density (Chapman et al., 2003; Gomez-Llano et al., 2018), have the potential to modulate male harm via both plastic and evolutionary responses. However, while such evidence suggests that male harm seems to be generally higher in environments to

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

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

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

Here, we test this idea by addressing whether adaptation to different temperatures results in the evolution of higher male harm in adapted vs. maladapted temperatures and, more importantly, whether pre-copulatory (behavioural, activity related) vs. copulatory (seminal fluid proteins) mechanism respond differently to cold vs. hot thermal regimes. To this aim, 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 populations under three different thermal regimes mimicking natural seasonal and circadian temperature variation. After 29-30 generations of experimental evolution, we 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 underlying pre- (male aggression and harassment levels) and copulatory (SFPs) traits.

Methods

Experimental evolution design

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

selected and of the same age, into a glass jar (16.5x19.5cm) containing two bottles with standard food. We allowed 6 days of interaction, collecting eggs on the 6th day that we raised at a standardized density (Clancy & Kennington, 2001) in bottles with 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, ignoring the cumulative harm effects over time that are typical of male harm and thus minimising selection for female resistance (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. Environmental conditions across all assays were controlled meticulously to ensure common garden conditions (see SI for full details).

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 compared reproductive success and survival of 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; Yun et al., 2021). For each population within each experimental evolution regime, we replicated these assays at 20, 24, and 28°C. 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 48h before starting the experiment, at which temperature they remained until the end of assays. We began experiments by placing virgin focal females (4-5d old) in individual vials containing medium with live yeast, after which we immediately added one (monogamy) or three (polygamy) experimental males from the corresponding population. On day 1 of the experiment, we

observed flies (Table 1) for 8h combining scan sampling with an all-occurrences recording rule (see SI) to score courtship intensity (courting males per female per hour), male-male aggression rate (aggressions per hour) and female rejection rate (rejections per hour; Bastock & Manning, 1955) 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 vials containing eggs at 24±4°C for 15-20d to allow F1 offspring emergence, and froze them 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 flies under these conditions for six weeks, during which time we recorded survivorship of focal females daily and replaced dead male flies with stock males maintained at each of the temperature treatments. Samples sizes for female reproductive success and survivorship are in the Table 1.

To explore male harm, we modelled reproductive success (sum of offspring for a female across the six weeks) 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. 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). Additionally, to further explore if overall harm was higher at adaptive vs. maladaptive temperatures, we performed a complementary

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

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

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

Proteomics assays (experiment 2)

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

virgin (first mating) and mated (successive matings) males across experimentally evolved regimes. We conducted assays at the common garden temperature of 24ºC (shared temperature across thermal regimes). Upon eclosion, we allocated virgin focal males into vials of 8 individuals until 4-5d-old. On the day of sample collection, we isolated 45 experimental females per population in vials, after which we immediately introduced focal males either into a female-containing (mated) or empty (virgin) vial. We flash-froze mated males in liquid nitrogen 25min after the start of mating, freezing a virgin male from the same population at the same time (see Hopkins et al., 2019a,b; Sepil et al., 2019). We repeated this procedure during two more consecutive days to obtain three independent biological replicates following a balanced design. 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 of the same temperature regime, mating status and replicate, which we sent for label-free quantitative proteomics sample preparation and quantification (protocol SWATH-MS; Gillet et al., 2012) at the SCISIE (University of Valencia) proteomics service (see SI for details). We thus analysed six samples per population (three virgin and three mated males), across four replicate populations per experimental evolution regime (i.e. n = 72). All assayed males across experimental evolution regimes were dissected at the same time (i.e. in balanced order across the same days) and proteomic analyses conducted at the same time for all samples, to avoid block effects.

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

For our analysis and visualization of abundance patters we averaged across 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 *NMF* package (Gaujoux & Seoighe, 2010). We identified seminal fluid proteins (SFPs) based on a high-confidence SFPs reference list from Sepil et al., (2019) and Wigby et al., (2020). We represented Venn diagrams on the number of proteins and percentage of SFPs using *ggvenn* package (Gao et al., 2021). Finally, to study differential evolution of SFPs, we repeated the elastic net regressions including only the SFPs dataset.

GxE assay (experiment 3)

To test for GxE interactions in male fitness 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, 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 with medium, after which we added two *spa*^{pol} males and one female (high-competition environment). We then placed four replicates (i.e. vials) per isoline under three different treatment temperatures (20, 24, and 28°C, ±4°C). We did not have enough flies to set up four replicates in 6 isolines (see Data), so we ended up with 114 replicates 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 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) package in RStudio.

Results

Harm is higher at evolved temperatures

Experimental evolution significantly modulated the degree to which high conflict hampered female reproductive success, with higher male harm in flies from the moderate regime (experimental evolution regime x mating system interaction: $F_{2,2939.1} = 3.04$, $P = 0.048$; Figs. 1 and S2). This was driven mainly by the fact that male harm was more constant (less plastic) in flies evolved in the moderate thermal regime, whereas it 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; experimental evolution regime x temperature treatment interaction: $F_{4,2939.5} = 2.89$, $P = 0.021$; Fig. 1, Table S3). 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. S3, Table S4a,b). Finally, direct comparison of male harm levels between adapted vs. non-adapted temperatures confirmed that, as predicted, overall male harm was higher at temperatures within vs. outside the thermal range at which flies had evolved (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 S3).

Male harassment decreases at colder temperatures

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, Table S2b,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 diversifies across thermal regimes, with SFPs characterising evolution at hot temperatures

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

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

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

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

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

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

Discussion

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 male pre-copulatory and copulatory sexual conflict traits and resulting male harm to females. Our results suggest 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 maintain standing genetic variation of secondary sexual traits (i.e. lek paradox), and c) how adaptation of male sexually selected traits in response to thermal ecology may foster diversification and reproductive barriers between populations.

First, we show that there is quick evolution of male harm to temperature after 29 generations of experimental evolution under different thermal regimes. We found higher levels of male harm (net impact of male exposure on female fitness) at temperatures at which flies had evolved to; 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 ancestral 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 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). This findings contribute to the growing body of evidence indicating that adaptation to novel environments can affect the level of sexual conflict (Martinossi-Allibert et al., 2018).

Second, we report strong evidence of fast divergent evolution of behavioural vs. sperm-related male traits involved in male harm at cold vs. hot regimes. Male harassment of females (pre-copulatory harm) evolved to be considerably less intense and thermally plastic in populations adapted to the cold regime. In contrast, seminal fluid proteins (SFPs) characterized the evolution of male seminal proteomes at hot vs. cold or moderate regimes (Figs. 3-5). This included proteins such as Semp1 and ovulin, which can be harmful to females (Wigby & Chapman, 2005). In fact, comparison of all SFPs across thermal regimes revealed that several proteins from the sex peptide and ovulin networks characterize evolutionary responses to the hot regime (Fig 5). This finding strongly suggests that temperature is likely to be a determining factor in the diversification of traits involved in male harm in *Drosophila* and, potentially, other ectotherms. The evolution of decreased male harassment at cold regime 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). Metabolic rate is intimately bound to temperature across the tree of life, particularly in ectotherms (Brown et al., 2004). Thus, cold temperatures may place a general constraint and/or simply increase the costs of male activity,

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

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

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

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

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

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

456 **Conclusions**

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

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 the differential expression of SFPs in males evolved at the hot regime, 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.

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

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

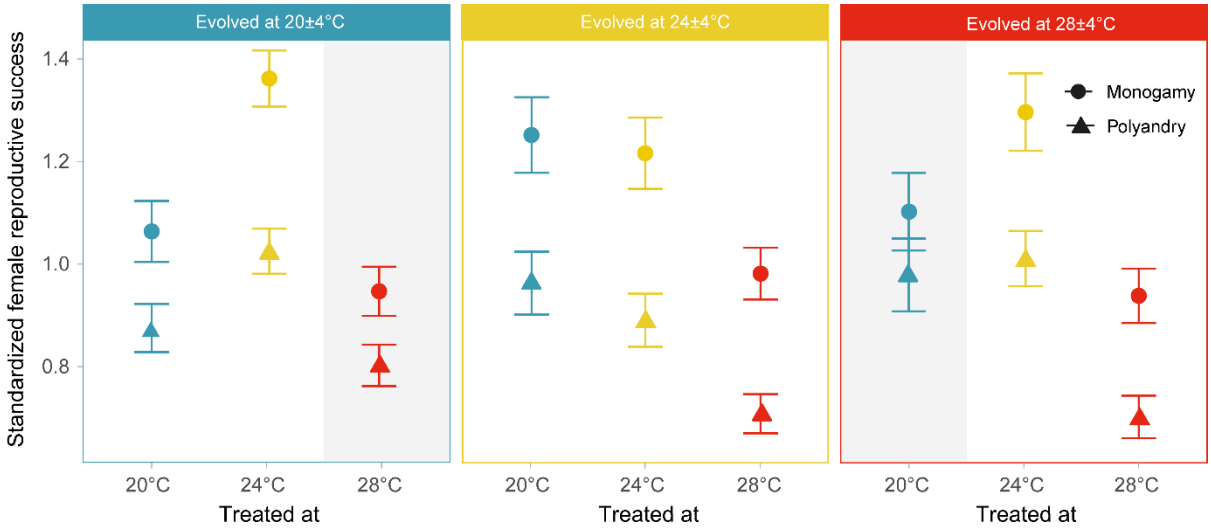

Figure 1 | Effect of mating system, temperature treatment and experimental evolution regime on female fitness. Female reproductive success (mean ± s.e. of four replicates) across mating systems (monogamy and polyandry), temperature treatments (20, 24 and 28°C) and experimental evolution thermal regimes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

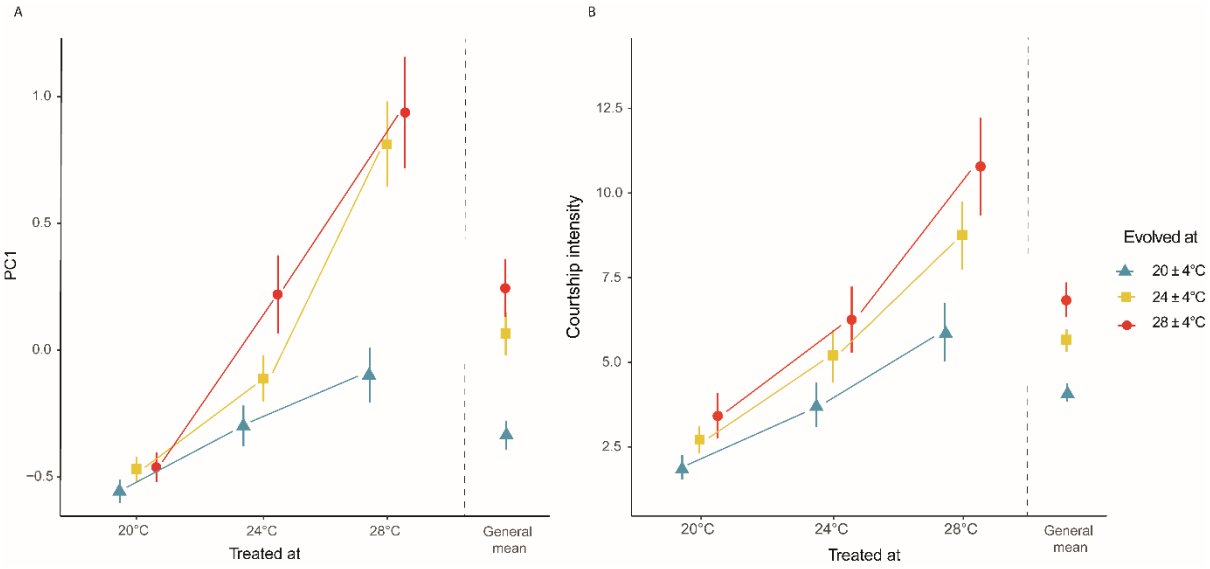

Figure 2 | Effect of temperature treatment and experimental evolution regime on pre-copulatory male harm. A) Mean (± s.e.) for PC1 from a PCA including behaviours causally related with pre-copulatory harm (courtship intensity, female rejection and male-male aggression), for assays at increased sexual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

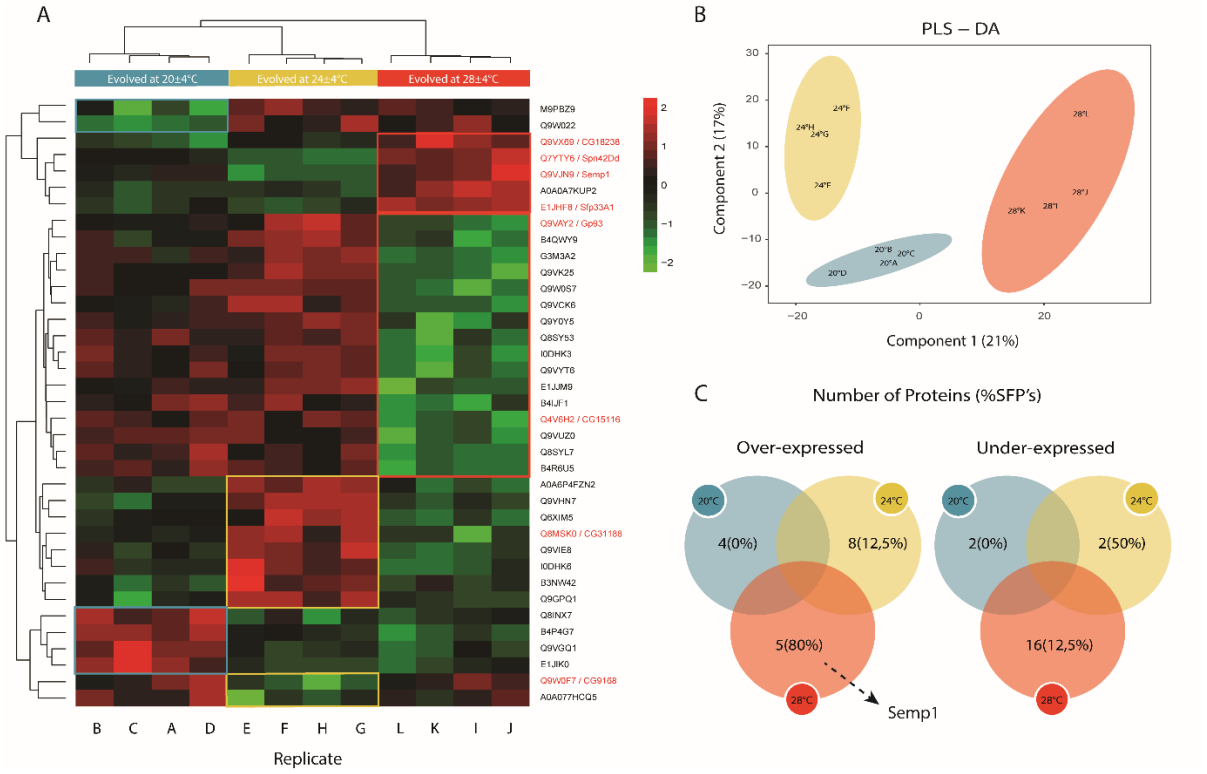

Figure 3 | Effect of experimental evolution regime on virgin males' seminal proteome. A) Heatmap showing the abundance of 37 proteins selected by the Elastic net regression. Each cell gives the across-biological replicate mean for that protein in each experimental evolution thermal regime and replicate.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

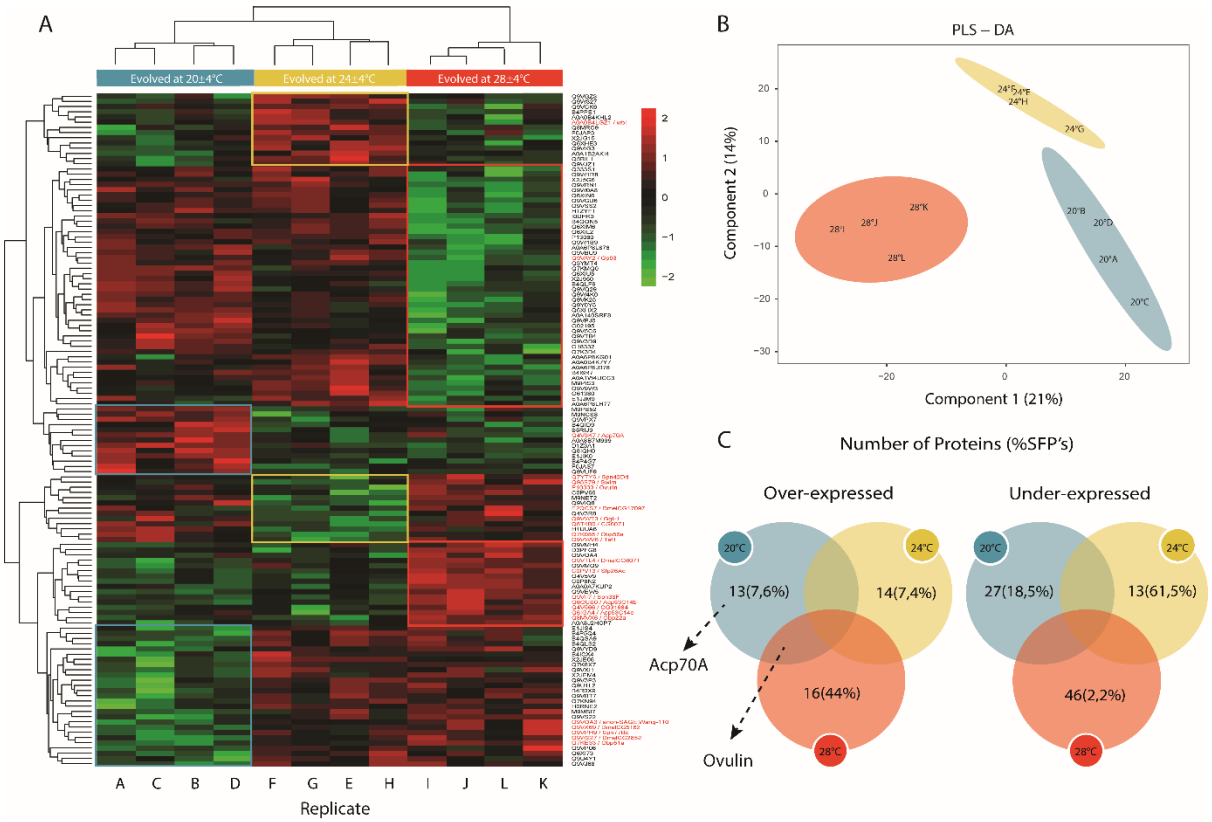

Figure 4 | Effect of experimental evolution regime on mated males' seminal proteome. A) Heatmap showing the abundance of 129 proteins selected by the Elastic net regression. Each cell gives the across-biological replicate mean for that protein in a given experimental evolution thermal regime and replicate.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

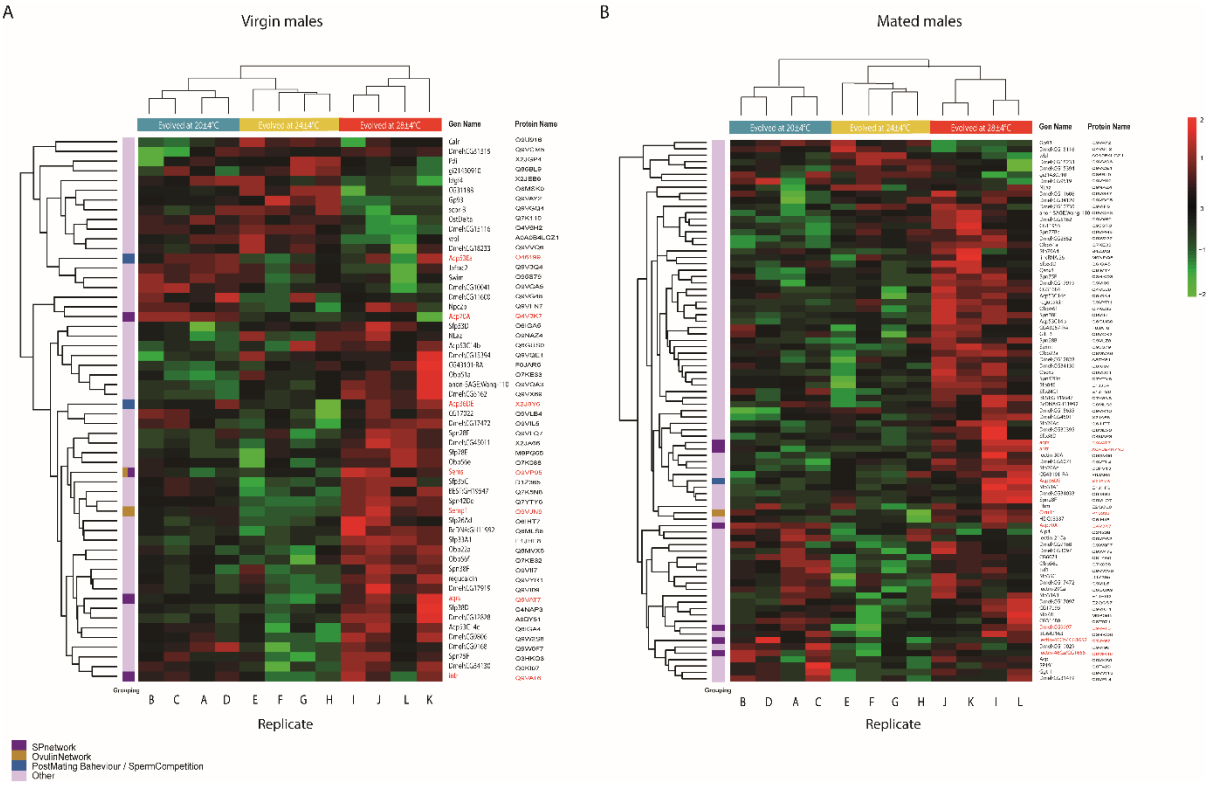

Figure 5 | Effect of experimental evolution regime on SFPs of virgin and mated males. A) Heatmap showing the abundance of 56 proteins selected by the Elastic net regression for virgin males. B) Heatmap showing the abundance of 85 proteins selected by the Elastic net regression for mated males. Each cell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

Supplementary Information

Materials and Methods

Experimental evolution design

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

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

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

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

75 Behavioural assays (experiment 1)

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

90 Male harm and behavioural analysis (experiment 1)

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

distribution with an "*identity"* as link function. For courtship and aggression rates, a zero inflated distribution was applied, while for female rejection rate, we used a negative binomial distribution with "*log"* as link function. Graphical inspection of the modelled component 1 of the PCA, revealed that the normality assumption was apparently violated. Natural logarithm transformation solved these problems and allowed us to run a LMM with a Gaussian error distribution and an "*identity"* as link function. 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. 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.

Proteomics assays (experiment 2)

Proteomics sample preparation

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

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

Spectral Library Building

Aliquots with an equivalent amount of a selection of samples were mixed to make a pool for building the spectral library (25 µg). The library electrophoresis was performed using a 12% precast gel (Bio-Rad) at 200V for 30 min. Gels were fixed with 40% ethanol/10% acetic acid for one hour and stained with colloidal Coomassie (Bio-Rad) for 15 min. Gels were destained with H_2O milliQ and cutted into six pieces for protein digestion.

In gel protein digestion

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

LC-MS/MS analysis

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

Protein Identification

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

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

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

SWATH analysis of individual samples

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

SWATH LC-MS/MS Analysis

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

7 to 40% B in 120 min (A: 0.1% FA; B: ACN, 0.1% FA) for at a flow rate of 300 nl/min. Peptides were analysed in a mass spectrometer nanoESI qQTOF (6600plus TripleTOF, ABSCIEX).

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

Protein quantification

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

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

Data analysis

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

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

GxE assay (experiment 3)

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. For reproductive success of focal males we used beta distribution with "*logit"* as link function.

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Results

Behaviorual assays (experiment 1)

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

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

277 Proteomics assays (experiment 2)

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

280

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

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

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

a)

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

b)

Experimental evolution regime	***PC1***				
	Effect	*F*	*Df*	*Df.res*	*P value*
20±4°C	Temperature treatment	11.71	2	347	**<0.001**
24±4°C	Temperature treatment	32.88	2	345	**<0.001**
28±4°C	Temperature treatment	27.21	2	347	**<0.001**

c)

Experimental evolution regime	***PC1***					
	Contrast	*Estimate*	*SE*	*df*	*t-value*	*P value*
20±4°C	20° – 24°	-0.31	0.117	1039	-2.64	**0.022**
	28° – 24°	0.19	0.117	1039	1.62	0.237
	28° – 20°	0.50	0.116	1039	4.30	**<0.001**
24±4°C	20° – 24°	-0.32	0.117	1039	-2.74	**0.017**
	28° – 24°	0.62	0.117	1039	5.29	**<0.001**
	28° – 20°	0.94	0.117	1039	8.03	**<0.001**
28±4°C	20° – 24°	-0.47	0.117	1039	-4.00	**<0.001**
	28° – 24°	0.47	0.116	1039	4.09	**<0.001**
	28° – 20°	0.94	0.117	1039	8.03	**<0.001**

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

a)

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

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

b)

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

c)

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

d)

Female reproductive success				
Effect	*F*	*Df*	*Df₂*	*P value*

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

313
314

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

a)

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

b)

Experimental evolution regime	***Lifespan***					
	Contrast	*Estimate*	*SE*	*Df_2*	*t-value*	*P value*
20±4°C	20° – 24°	-0.48	0.08	Inf	-5.96	**<0.001**
	20° – 28°	-0.87	0.08	Inf	-10.67	**<0.001**
	24° – 28°	-0.38	0.08	Inf	-4.86	**<0.001**
	Polyandry - Monogamy	-1.11	0.07	Inf	-16.430	**<0.001**
24±4°C*	20° – 24°	-0.80	0.09	Inf	-9.31	**<0.001**
	20° – 28°	-1.52	0.09	Inf	-17.59	**<0.001**
	24° – 28°	-0.72	0.08	Inf	-9.04	**<0.001**
	Polyandry - Monogamy	-1.37	0.07	Inf	-19.863	**<0.001**
28±4°C	20° – 24°	-0.89	0.08	Inf	-10.35	**<0.001**
	20° – 28°	-1.67	0.08	Inf	-19.48	**<0.001**
	24° – 28°	-0.78	0.08	Inf	-9.68	**<0.001**
	Polyandry - Monogamy	-1.27	0.07	Inf	-18.268	**<0.001**

c)

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

d)

Lifespan						
Temperature treatment	*Contrast*	*Estimate*	*SE*	*Df_2*	*t-value*	*P value*
20°C	Polyandry - Monogamy	-1.22	0.07	Inf	-16.984	**<0.001**
24°C		-1.40	0.07	Inf	-20.054	**<0.001**
28°C		-1.12	0.06	Inf	-17.225	**<0.001**

e)

Lifespan			
Effect	*Chisq*	*Df*	*P value*
Experimental evolution regime x temperature treatment x mating system	8.006	4	0.091

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

	20 ± 4°C		24 ± 4°C		28 ± 4°C			
	V	**M**	**V**	**M**	**V**	**M**	**Molecular function**	**Biological process**
A0A0B4LGZ1				+				Probable chaperone protein involved in dorsoventral axis patterning in early embryos. Probably acts by folding and targeting pipe into the Golgi.
C0PV13						+		Sexual reproduction
E1JHF8					+			Sexual reproduction
E2QCS7				–			Serine hydrolase activity, triglyceride lipase	Lipid metabolic process, sexual reproduction.
P10333				–			Identical protein binding	Mating, positive regulation of octopamine signalling pathway, positive regulation of ovulation, sexual reproduction, sperm competition
Q4V3K7		+					Hormone activity	Regulation of female receptivity, post-mating behaviour
Q4V566						+	Lipase activity	Lipid catabolic process
Q4V6H2					–		Peroxidase activity, thioredoxin-dependent peroxiredoxin activity	Response to oxidative stress, sexual reproduction
Q6IGA4						+		Sexual reproduction.
Q6GUS0						+		Sexual reproduction
Q7K088				–			Odorant binding	Sensory perception of smell, sensory perception of chemical stimulus, sexual reproduction
Q7KE33		–					Odorant binding	Sensory perception of smell, sensory perception of chemical stimulus, sexual reproduction
Q7YTY6				–	+			Serine protease inhibitor with activity toward trypsin. Involved in innate immunity to fungal infection by negatively regulating the Toll signalling pathway and suppressing the expression of the antifungal peptide drosomycin. Acts upstream of SPE and grass, and downstream of the fungal cell wall pattern recognition receptor GNBP3. May function specifically in the GNBP3-dependent beta-1,3-glucan branch of the Toll pathway.
Q8MSK0			+				Iron ion binding, L-ascorbic acid binding, rocollagen-proline 4-dioxygenase activity	Peptidyl-proline hydroxylation to 4-hydroxy-L-proline, sexual reproduction
Q8MVX6						+	Odorant binding	Sensory perception of chemical stimulus, sexual reproduction
Q8T4B0				–			Metalloaminopeptidase activity, peptide	Peptide catabolic process, proteolysis, sexual reproduction

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

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

a)

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

b)

Experimental evolution regime	***Aggression rate***					
	Contrast	*Estimate*	*SE*	*Df*$_2$	*t-value*	*P value*
20±4°C	20° – 24°	0.16	0.33	1037	0.50	0.869
	28° – 24°	0.09	0.26	1037	0.37	0.924
	28° – 20°	-0.07	0.30	1037	-0.21	0.974
24±4°C	20° – 24°	-0.78	0.26	1037	-2.95	**<0.001**
	28° – 24°	0.36	0.17	1037	2.09	0.091
	28° – 20°	1.14	0.23	1037	4.88	**0.009**
28±4°C	20° – 24°	-0.83	0.29	1037	-2.87	**0.011**
	28° – 24°	0.07	0.16	1037	0.49	0.878
	28° – 20°	0.91	0.28	1037	3.30	**0.002**

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

a)

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

b)

Experimental evolution regime	***Rejection rate***					
	Contrast	*Estimate*	*SE*	*Df*$_2$	*t-value*	*P value*
20±4°C	20° – 24°	-1.47	0.42	2116	-3.54	**0.001**
	28° – 24°	0.50	0.38	2116	1.34	0.374
	28° – 20°	1.98	0.42	2116	4.77	**<0.001**
24±4°C	20° – 24°	-0.39	0.39	2116	-1.00	0.573
	28° – 24°	0.31	0.37	2116	0.83	0.680
	28° – 20°	0.70	0.39	2116	1.82	0.164
28±4°C	20° – 24°	-2.15	0.40	2116	-5.32	**<0.001**
	28° – 24°	0.18	0.36	2116	0.52	0.862
	28° – 20°	2.34	0.40	2116	5.81	**<0.001**

c)

Experimental evolution regime	***Rejection rate per courtship***			
	Effect	*Chisq*	*Df*	*P value*

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

373
374 d)

Experimental evolution regime	***Rejection rate per courtship***					
	Contrast	*Estimate*	*SE*	*Df_2*	*t-value*	*P value*
20±4°C	20° – 24°	0.76	0.40	2117	1.91	0.134
	28° – 24°	0.11	0.26	2117	0.43	0.900
	28° – 20°	-0.65	0.39	2117	-1.69	0.207
24±4°C	20° – 24°	0.04	0.29	2117	0.16	0.986
	28° – 24°	-0.28	0.21	2117	-1.34	0.375
	28° – 20°	-0.33	0.27	2117	-1.24	0.427
28±4°C	20° – 24°	1.56	0.35	2117	4.48	**<0.001**
	28° – 24°	0.22	0.16	2117	1.36	0.361
	28° – 20°	-1.34	0.34	2117	-3.91	**<0.001**

375

376 e)

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

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

a)

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

b)

Temperature treatment	***Courtship rate***				
	Estimate	*SE*	*Df_2*	*t-value*	*P value*
20°C	-0.09	0.08	2110	-1.033	0.301
24°C	-0.20	0.08	2110	-2.520	**0.012**
28°C	-0.53	0.07	2110	-7.495	**<0.001**

c)

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

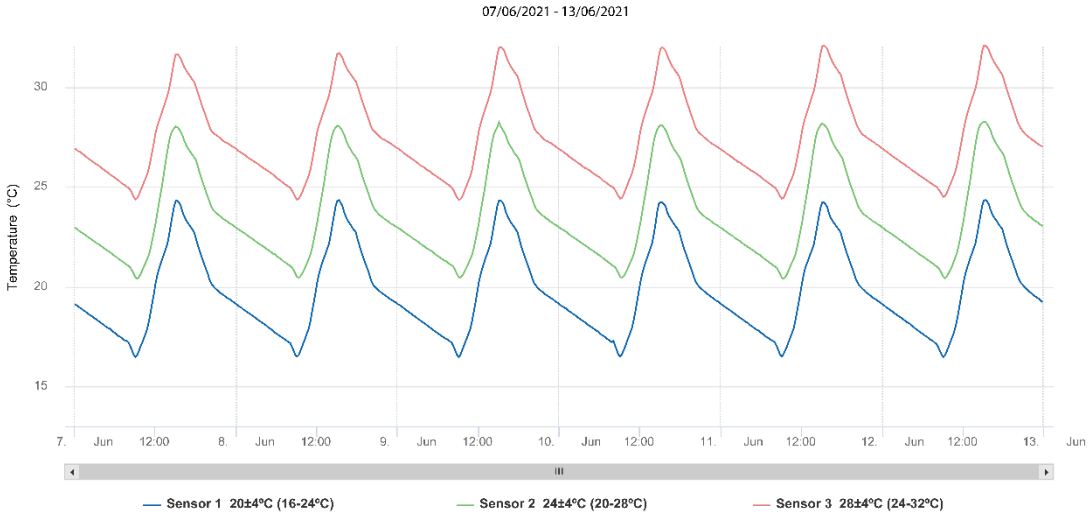
Table S9. Estimates of the explained variance by the fixed effects only for all full models. For rejection and courtship rates, we ran zero-inflated models, and the explained variance was calculated accordingly. Similarly, for the rejection per courtship rate we ran a binomial model.

Variable modelled	Explained variance (R^2m)
PC1	0.1483
Female reproductive success	0.0849
Lifespan	0.0358*
Rejection rate	delta (0.6106) lognormal (0.8928) trigamma (0.0965)
Rejection per courtship rate	theoretical (0.8773) delta(0.7642)
Courtship rate	delta (0.1101) lognormal (0.1275) trigamma (0.0924)

*Using Pseudo-R^2

Table S10. Parameters used for Elastic net analysis.

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



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

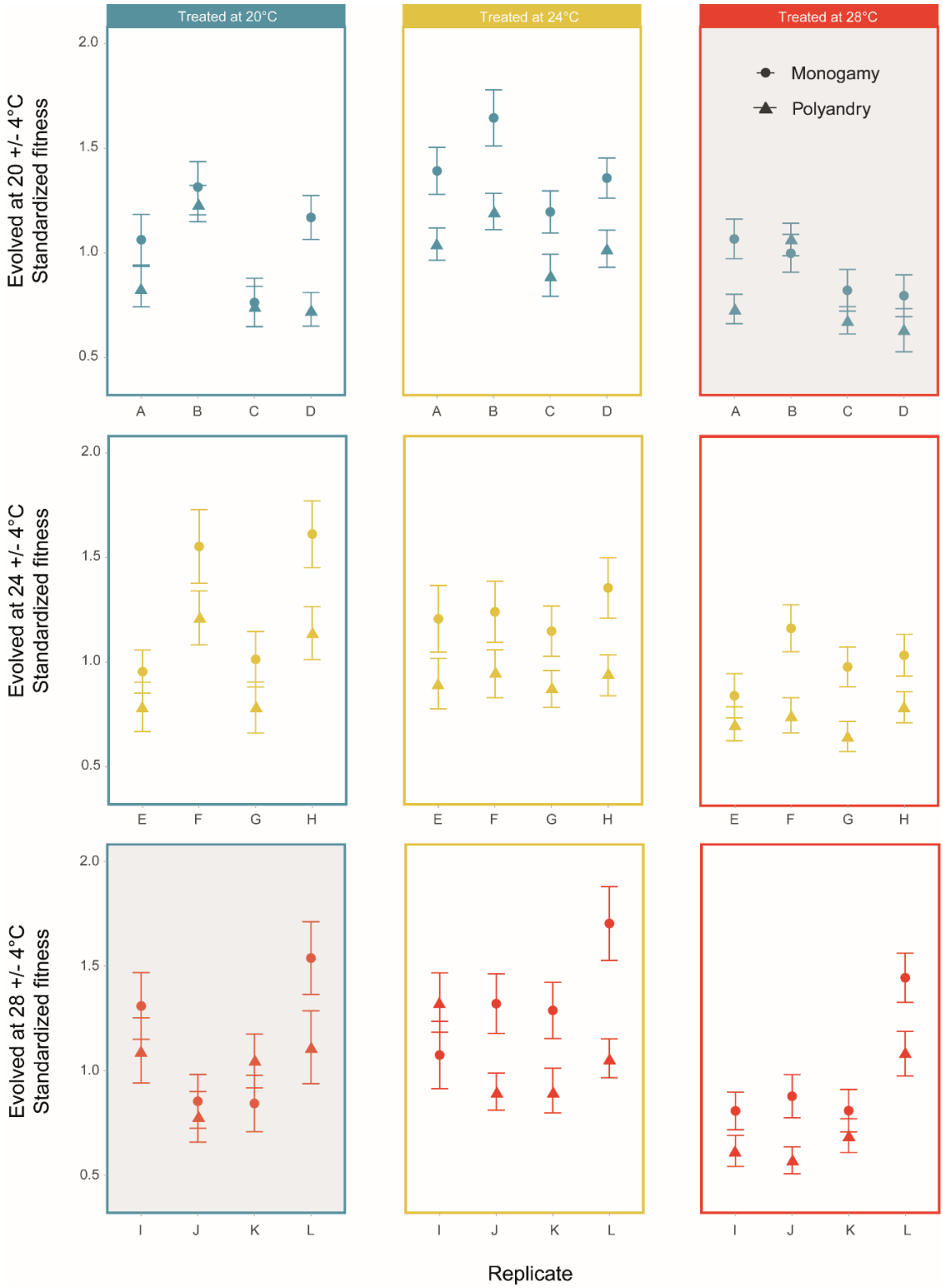


Figure S2 | Effect of mating system, temperature treatment and experimental evolution regime on female fitness by replicate. Female reproductive success (mean ± s.e.) across treatments and replicates. Data were standardized for each experimental evolution regime by dividing each value by the mean of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

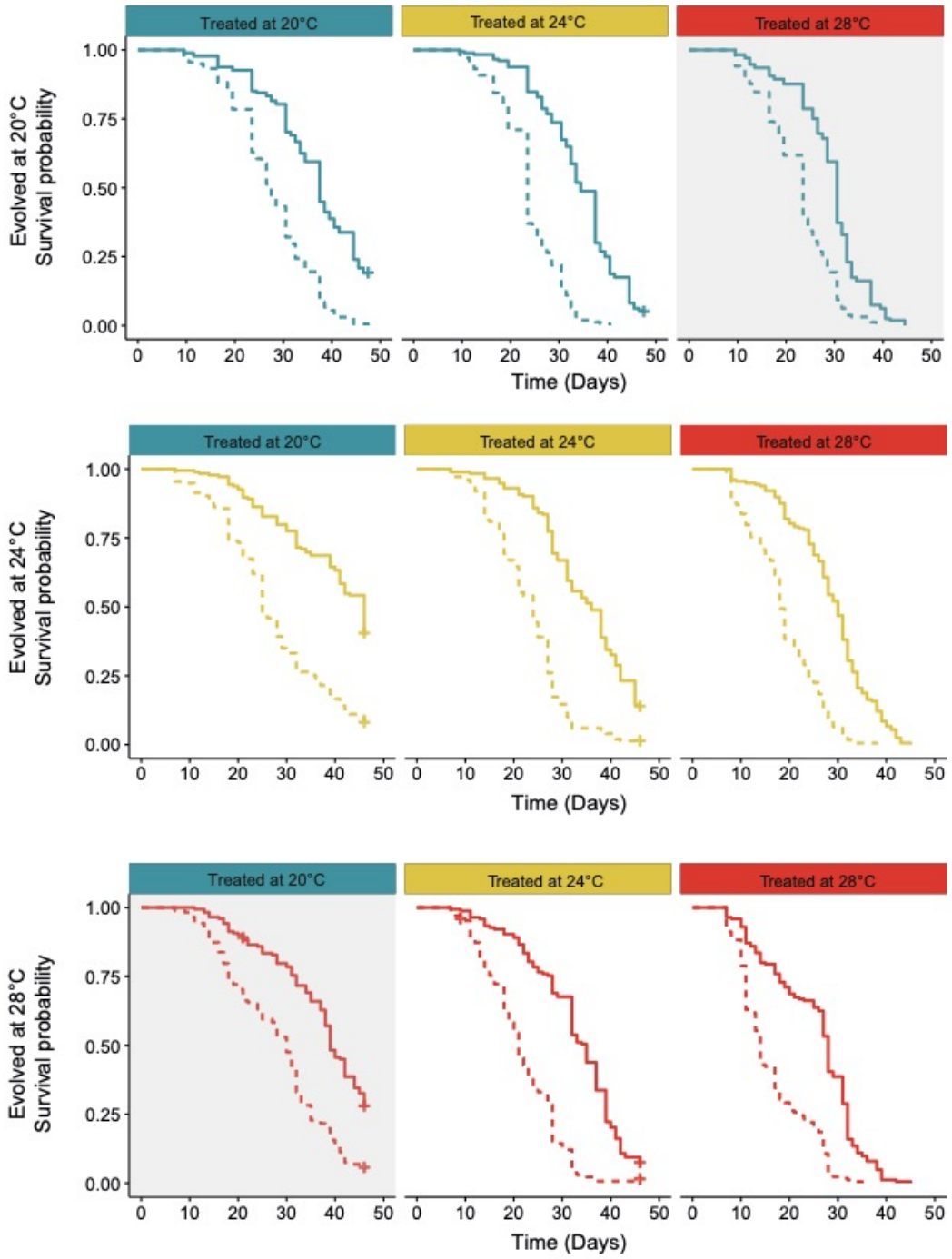
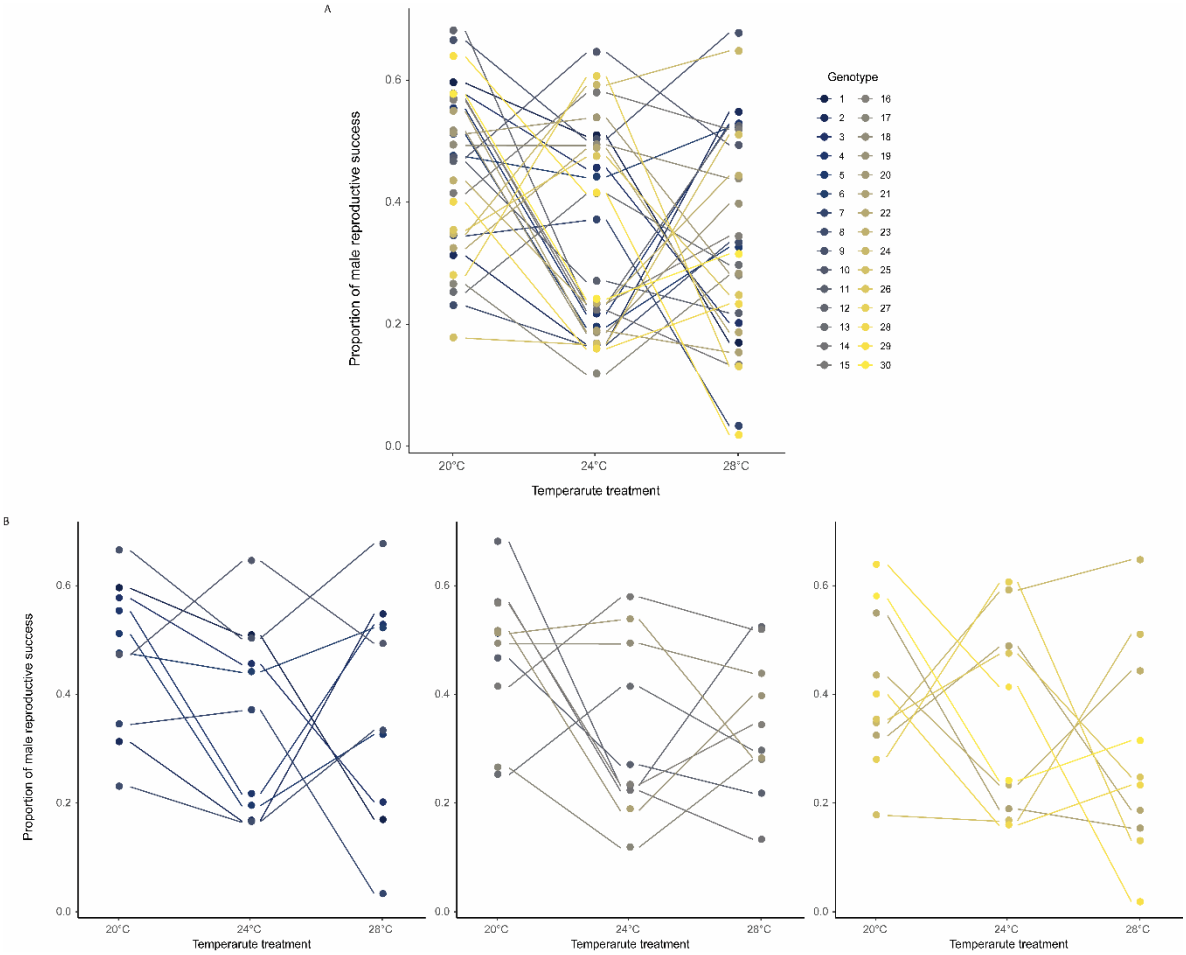


Figure S3 | Effect of mating system, temperature treatment and experimental evolution regime on female survival. Female survival (four replicates) across mating systems, temperature treatments and experimental evolution thermal regimes. The difference between female survival kept in monogamy (solid lines) vs. polyandry (dashed lines), was higher when flies were treated at temperatures within the thermal regime of evolution, compared to those outside this range (shaded panels).



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425 **Figure S4 | Genotype-by-environment interactions for male reproductive success**. A) Reaction
426 norms for male reproductive success in 30 genotypes analysed across three temperature treatments.
427 B) Reaction norms for male reproductive success split into 3 panels to clearly illustrate each line.

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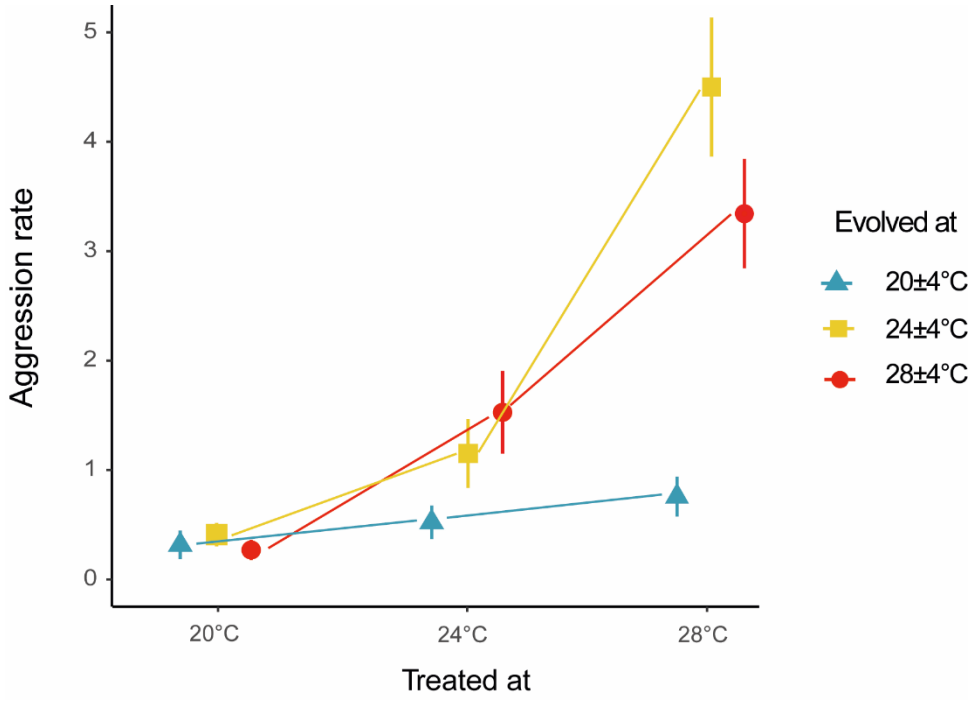

Figure S5 | Effect of temperature treatment and experimental evolution regime on male-male aggression rate. Aggressions male-male per hour (mean ± s.e.; four replicates) across temperature treatments and experimental evolution thermal regimes.

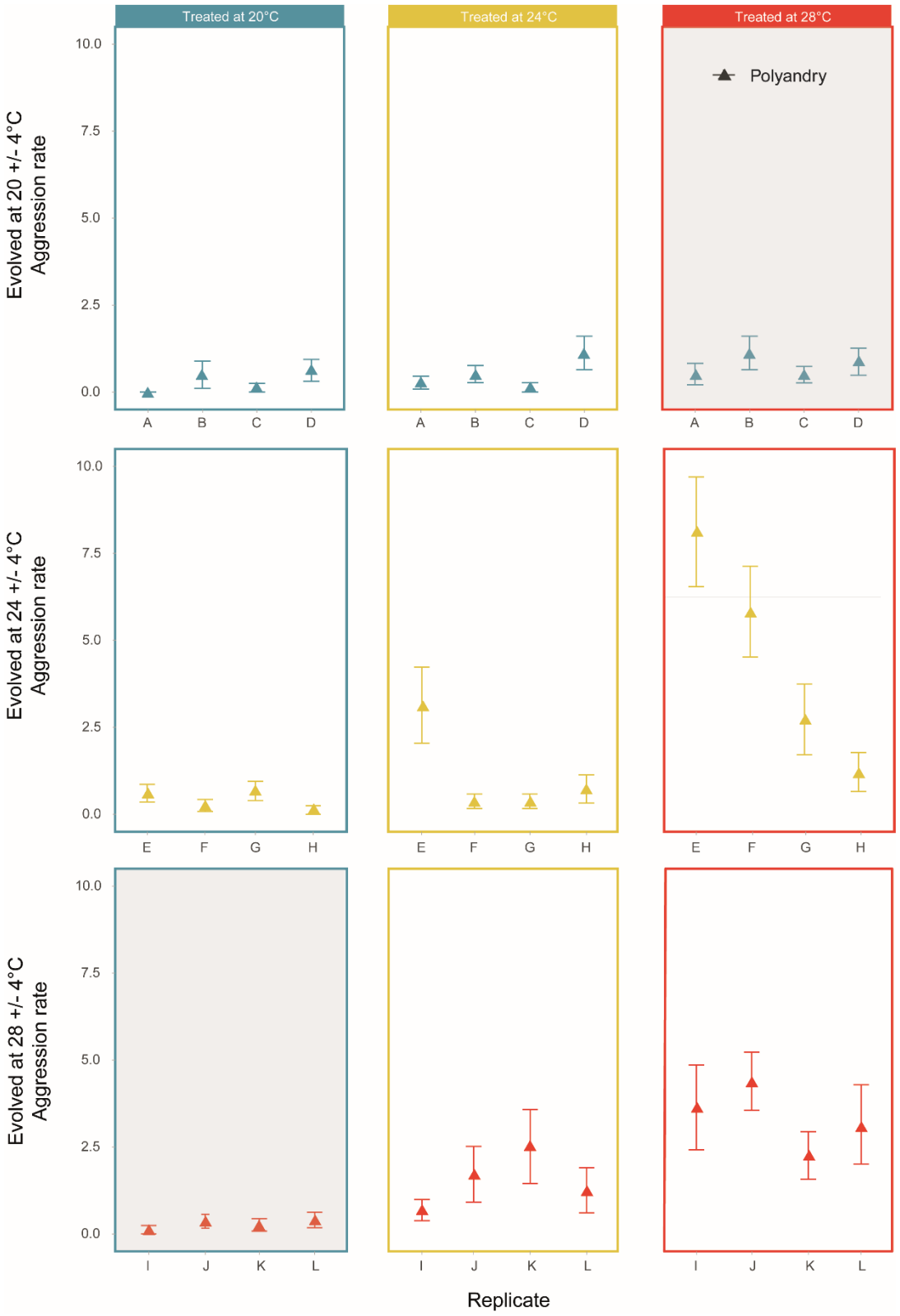

Figure S6 | Effect of temperature treatment and experimental evolution regime on male-male aggression rate by replicate. Aggressions male-male per hour (mean ± s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the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

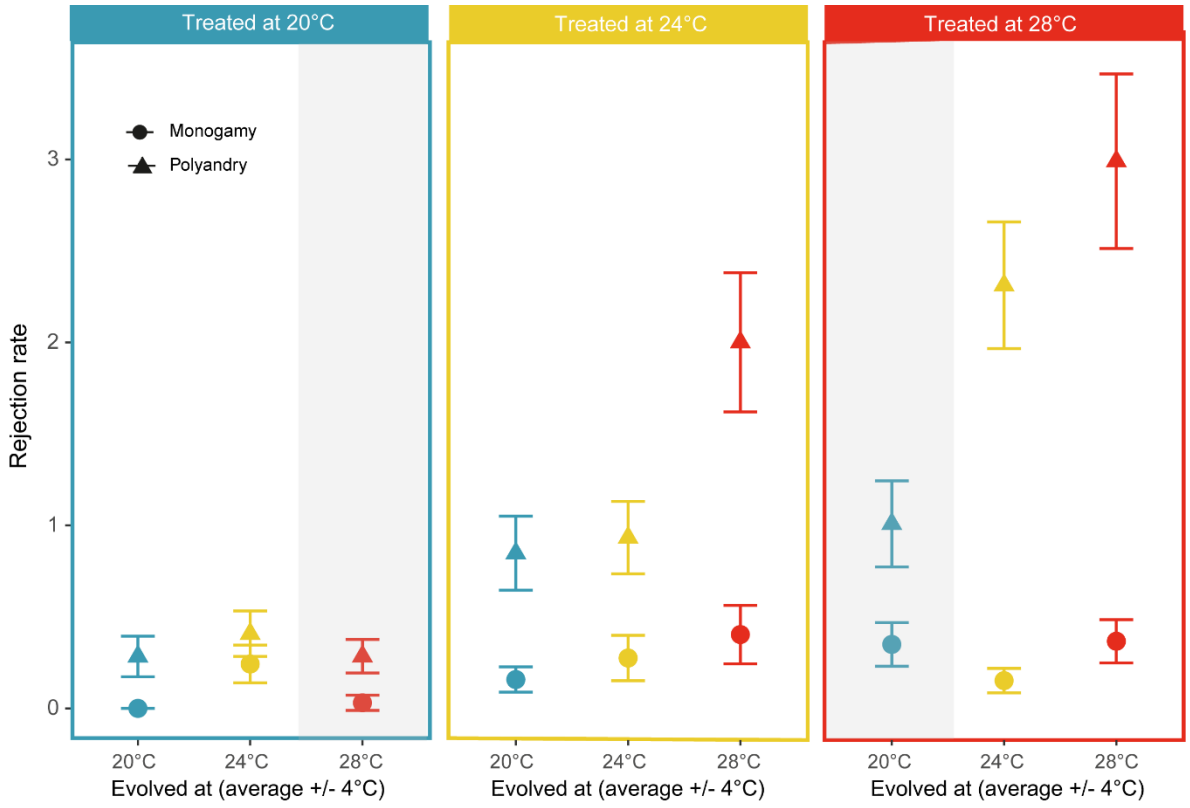

Figure S7 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate. Female rejections per hour (mean ± s.e.; four replicates) across temperature, mating system treatments and experimental evolution thermal regimes. Shaded panels denoted temperature treatments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

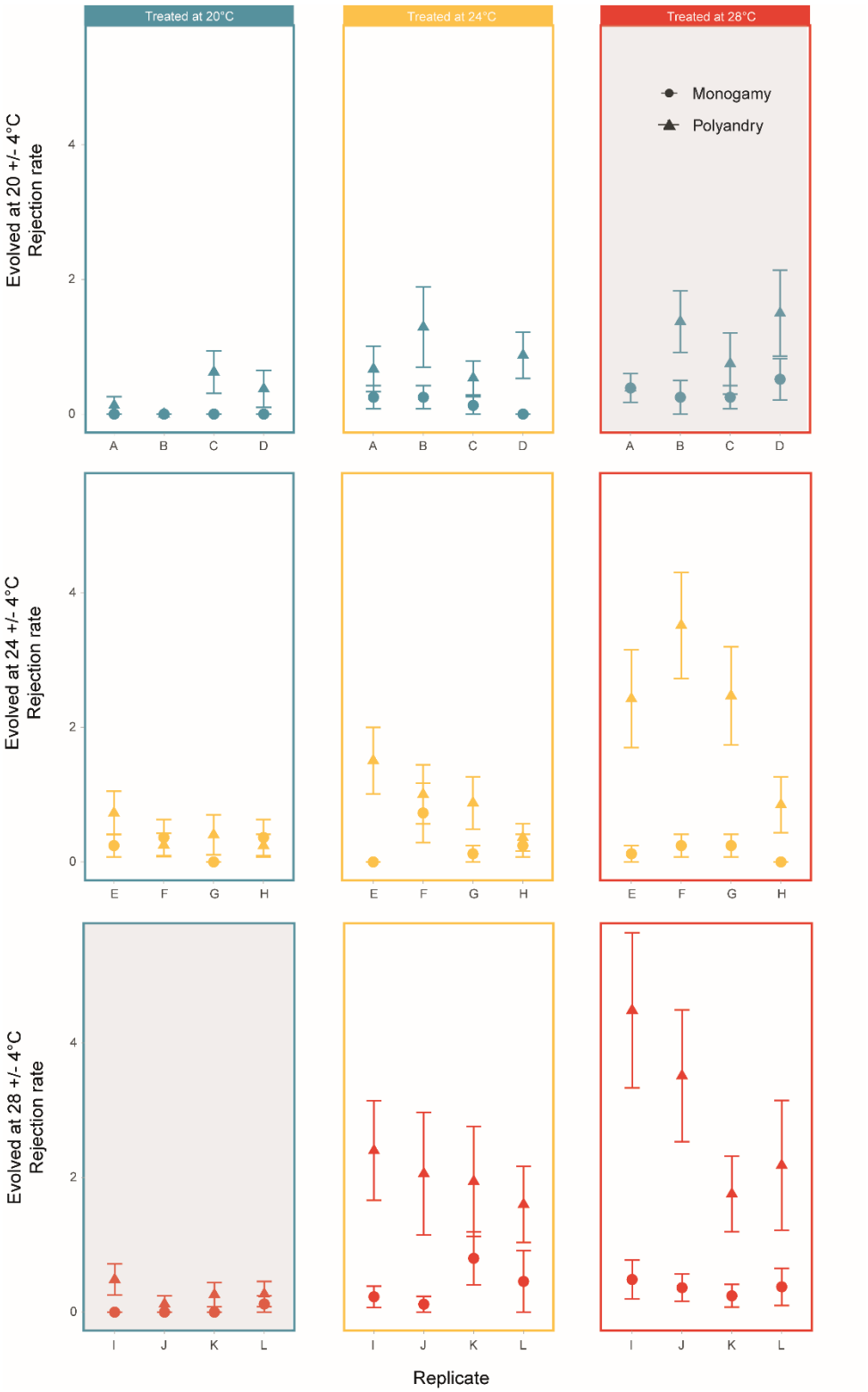

Figure S8 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate by replicate. Female rejections per hour (mean ± s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime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

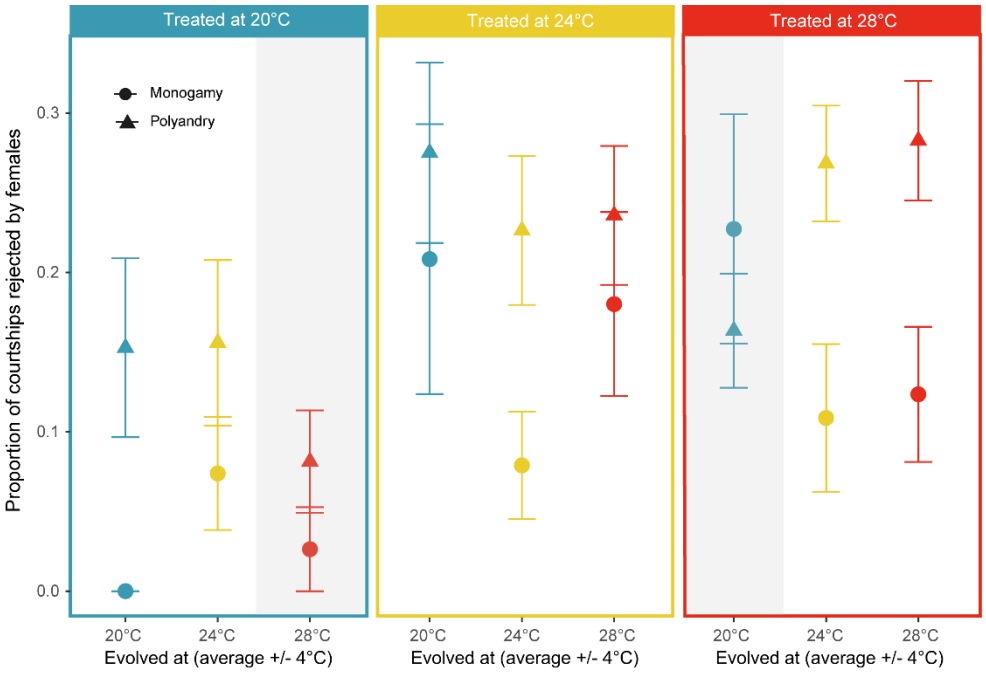

Figure S9 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate per courtship. Proportion of courtships rejected by females (mean ± s.e.; four replicates) across temperature, mating system treatments and experimental evolution thermal regimes. Shaded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

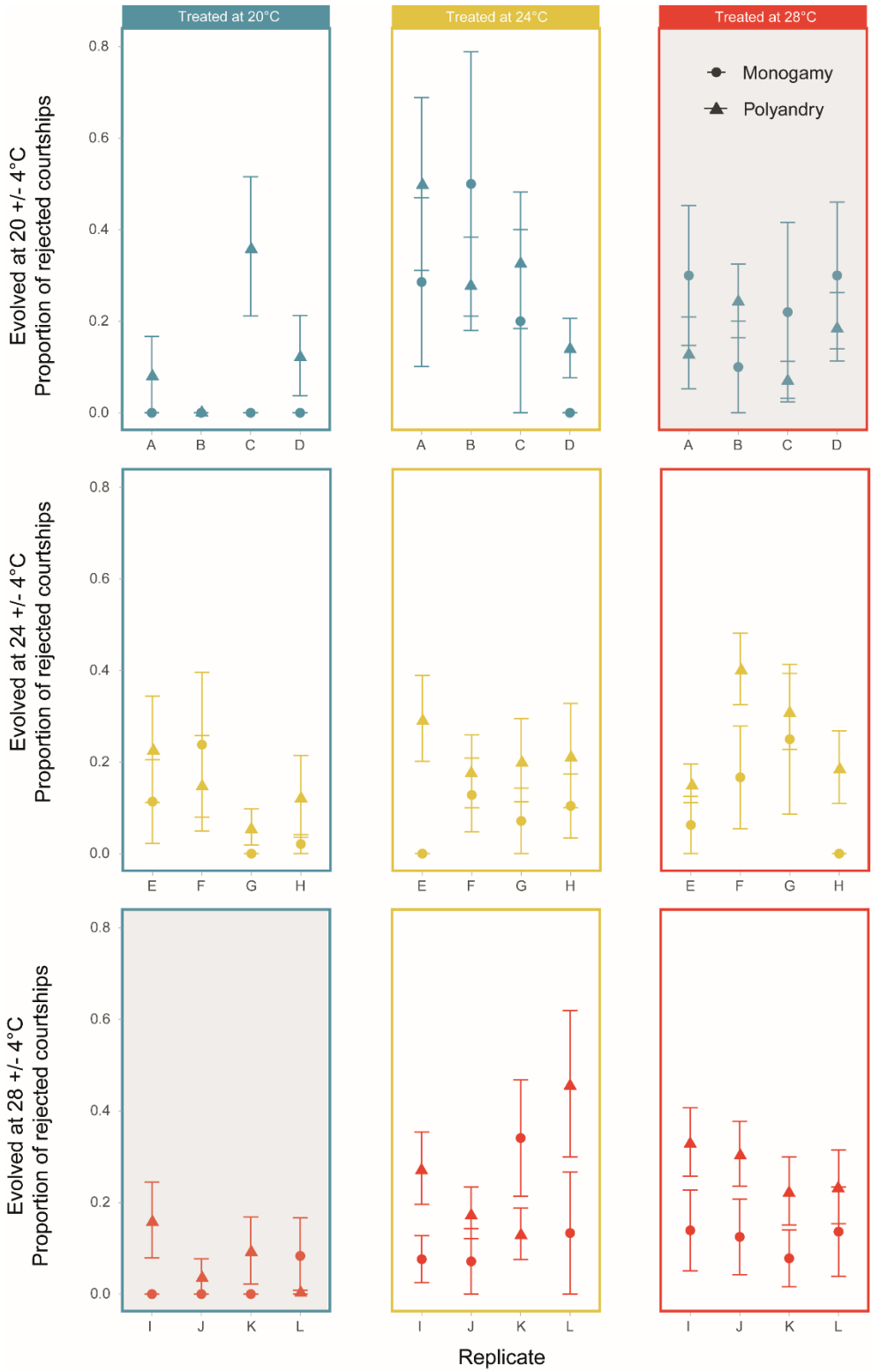

Figure S10 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate per courtship by replicate. Proportion of courtships rejected by females (mean ± s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental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

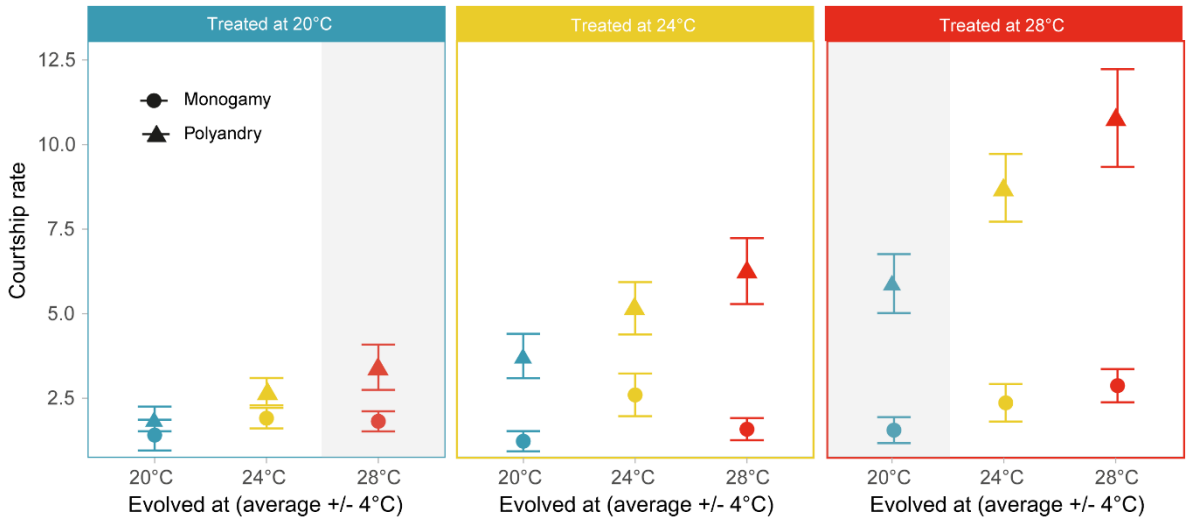

Figure S11 | Effect of mating system, temperature treatment and experimental evolution regime on courtship rate. Courtship per female per hour (mean ± s.e.; four replicates) across temperature, mating system treatments and experimental evolution thermal regimes. Shaded panels denoted temperature treatments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

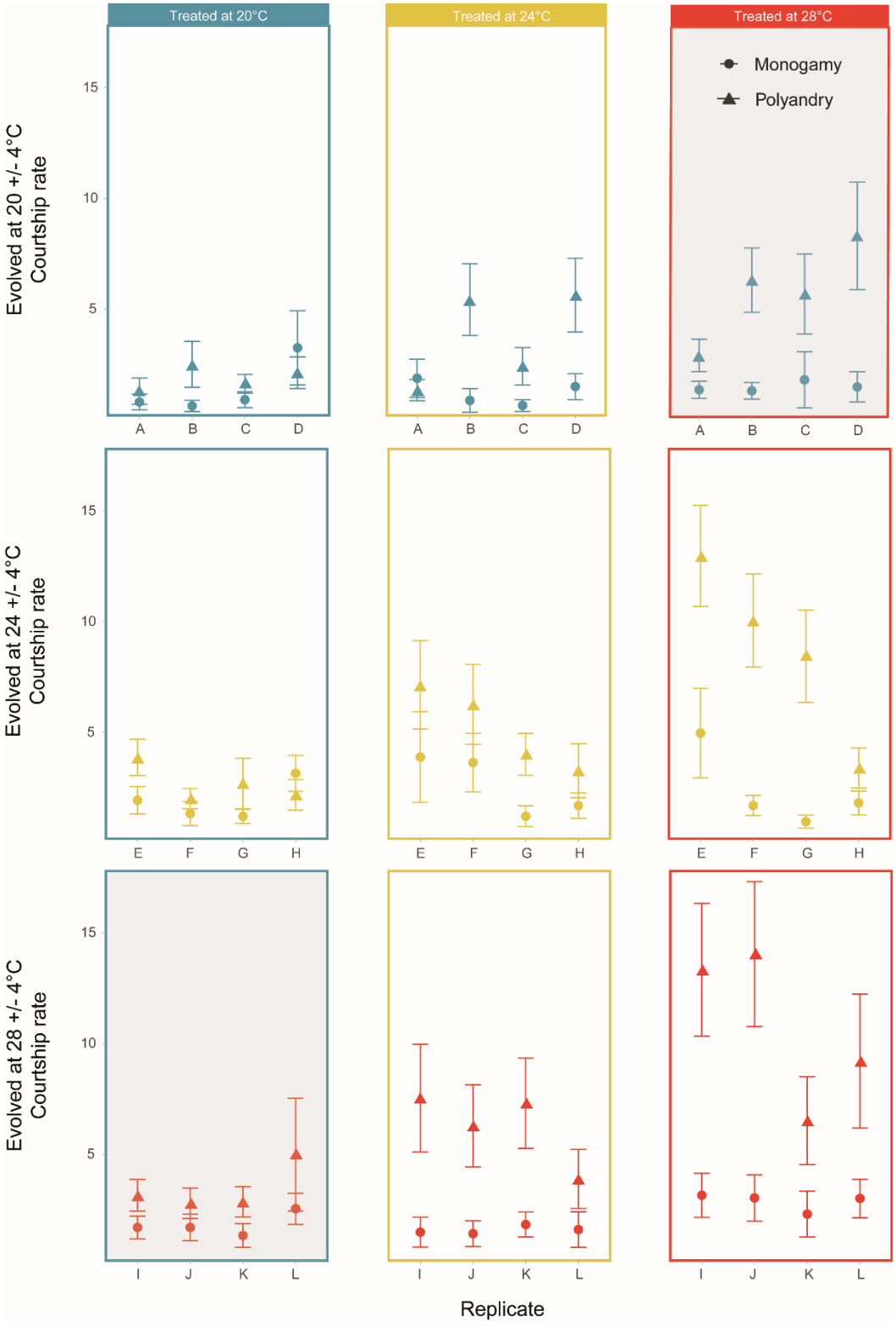

Figure S12 | Effect of mating system, temperature treatment and experimental evolution regime on courtship rate by replicate. Courtship per female per hour (mean ± s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for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