Temperature drives the divergent evolution of male harm to females

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ABSTRACT

Strong sexual selection promotes population viability and evolvability, but sexual conflict can offset such benefits. Male harm adaptations leading to pre-copulatory (i.e. harassment) and/or copulatory (i.e. traumatic insemination) harm to females are taxonomically widespread, depress population growth, and can affect the dynamics of adaptation and evolutionary rescue, but we largely ignore what factors modulate their evolution. Here, we show that temperature drives the divergent evolution of male harm in *Drosophila melanogaster*, a model species in the study of sexual conflict that exhibits both intense harassment of females and copulatory harm via seminal fluid proteins (SFPs). After 30 generations of experimental evolution of wild flies under cold (20±4ºC), moderate (24±4ºC) and hot (28±4ºC) thermal regimes mimicking natural conditions in the wild (average ± circadian range), we characterized overall levels and thermal plasticity of harm and male harassment behaviours, as well as the seminal proteome. Quick local adaptation was evidenced by higher levels of male harm at evolved (vs. non-evolved) temperatures. Furthermore, pre-copulatory vs. copulatory mechanisms of harm responded divergently to temperature. Male harassment was substantially lower and less plastic in lines evolved at cold temperatures, while the seminal proteome of lines evolved at warm temperatures was characterized by the differential expression of SFPs. Such quick divergent evolutionary responses suggest high levels of standing genetic variation in the population of origin. Accordingly, we derived isogenic lines from the ancestral wild population and show strong quadratic GxE interactions for male reproductive success across studied temperatures. Our results suggest: a) that temperature can be key to understand past and future (e.g. global warming) evolution of sexual conflict and the net effects of sexual selection on populations, and b) that natural temperature fluctuations can maintain high levels of standing genetic variation for male harm traits, contributing to resolve the lek paradox.

Main

Sexual selection can improve population viability and evolvability, making populations better able to adapt to a changing environment[1–4]. Driven by competition for mates and their gametes, sexual selection is widespread and important in both females and males[5]. Nevertheless, anisogamy commonly results in asymmetries in the strength and form of sexual selection across the sexes[6]. 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[1,2,4]. However, the same divergent selective pressures that make of sexual selection such an effective evolutionary sieve set the scene for sexual conflict, scenarios where female and male evolutionary interests misalign[7]. Actually, in sexually reproducing organisms the evolutionary interests of the sexes rarely coincide[8]. Alleles that confer a reproductive advantage to one sex may have opposing effects in the other, so that ensuing reproductive strategies evolve against each other[9,10]. 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[8,11–13].

Harmful male adaptations to females (male harm) are incredibly pervasive, diverse and sophisticated across the tree of life[8]. On the one hand, male harassment of females during pre-copulatory competition for mating has been documented in many vertebrate and invertebrate species[14]. 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[15] to adaptations for traumatic insemination that include seminal darts[16], spiny penises and even genital ablation[17,18]. Male harm thus drives antagonistic female-male co-

evolution in a host of behavioural and morphological traits[8], and may even act as an engine of speciation[19,20]. 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[14], even to the point of facilitating extinction[21]. Recent theoretical models suggest that such negative effects may compound when harmful traits are linked to condition[22], in which case good-genes selection can feed back to intensify male harm[23] and male harm can slow down evolutionary rescue[14]. 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[24]. Ecology has been shown to play a central role in shaping patterns of population divergence via sexual conflict[25,26], as well as in determining the intensity of male harm and to what degree it may offset good genes selection[27–29]. 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[29,30], suggesting that temperature may be a key player in male harm evolution[31].

To test this idea, we collected *D. melanogaster* from a wild population that has been shown to be thermally plastic for male harm[29] and set up 12 experimental evolution lines (N = 200; 4 replicates per treatment) under either cold (20±4ºC), moderate (24±4ºC) or hot (28±4ºC) thermal regimes mimicking natural seasonal and circadian temperature variation. *D. melanogaster* is the main 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[32–34]. 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[15,35]. 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[36–38], suggesting they are sensitive to environmental variation. After 29 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 common-garden behavioural, fitness and seminal proteome assays at 24°C 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.

Results

Harm to females is higher at evolved temperatures.

First (experiment 1), we measured reproductive success and survival of experimentally evolved female flies from each population, during six weeks, at 20, 24, or 28°C and under low conflict (i.e. monogamy; one male and one female per vial) vs. high conflict (i.e. polyandry; three males and one female per vial). This is standard procedure to gauge male harm in *Drosophila*, where these sex ratios represent biologically relevant scenarios [14,39,40]. Our experimental evolution procedure selected for early-life reproduction (see methods for details), thus erasing the late-life cumulative effects of harm on females and minimizing selection on female resistance to harm. Under these conditions, 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 S1). 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. 2 and Table S2a, b). Finally, direct comparison (see

methods) 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 2).

Cold temperatures decrease the levels and thermal plasticity of male harassment.

Next, we investigated whether pre-copulatory male harm mechanisms, and their thermal plasticity, were affected by experimental evolution regimes. We measured male-male aggression, courtship intensity and ensuing female rejection rates (rejection of a courting male by extrusion of the vaginal plates and/or kicking the male[41]) for 8hr during the first day of experiment 1. Previous studies in this species have shown that male harm is directly related to courtship intensity, female rejection and male intrasexual competition via male-male aggression[32,34,42]. To study the joint contribution of these behaviours to pre-copulatory male harm under high sexual conflict (male-male aggression can only happen under the polyandry treatment), we performed a PCA. PC1 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 S3a), so we took PC1 as an overall index of male harassment to females. 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. 3 and Table S3b,c) in flies evolved in the cold thermal regime . Analyzing these three behaviours separately, as well as effects across mating systems, confirmed these results (see methods and SI).

Thermal regimes shape the evolution of the male seminal proteome, with SFPs responding differentially to hot temperatures.

To study whether and how the seminal fluid of males evolves in response to temperature, we set up a series of assays (experiment 2) and conducted label-free quantitative proteome analysis of the accessory glands of mated and virgin males[37,38,43] across experimentally evolved lines. All assays were conducted at the common garden temperature of 24ºC (see methods), which was the shared temperature in all three thermal evolution regimes. We found a total of 1452 proteins, 149 of which have been priorly identified as SFPs. We analyzed virgin and mated data sets independently using a multivariate elastic net regression (see methods), 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. 4a), with each cluster including the four replicates within each regime. A partial least-squares discriminant analysis (PLS-DA) supported these findings (Fig. 4b), 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.4c). 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. 4c and Table S7). 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[44,45].

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. 5a), which was also confirmed by the PLS-DA analysis (Fig. 5b). 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 5c and Table 7S). 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 the ancestral wild population set the scene for quick adaptive responses.

Finally, we conducted a series of fitness assays (experiment 3) across male genotypes (i.e. isogenic lines) derived from wild-caught flies from the ancestral wild population (see methods) to test for the existence of GxE interactions within the range of temperatures at which reproduction is optimal for this population in the wild (20-28ºC). We set up isogenic

lines and examined male reproductive success of focal males from each isoline at 20, 24 and 28ºC (three or four replicates per isoline per temperature treatment). In each assay, a focal male had to compete for reproduction against two rival spa^{pol} males (i.e. with a recessive eye-colour genetic marker) over access to a spa^{pol} female in a vial until its death, mimicking the high male-male competition and sexual conflict scenario in experiment 1. The use of spa^{pol} rivals/mates backcrossed against the same genetic background as focal flies allowed us to estimate the reproductive success of males as the proportion of sired offspring vs. offspring sired by rival males. Importantly, we regularly changed rivals/mates so that focal males competed over access to different females against different males during their lifespan (see methods), mimicking competition across different mating patches in the wild. 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. 6).

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 local adaptation 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. At the same time, 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[29]. 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 local adaptation, given that strong sexual selection in males has led to the evolution of male harm in this species[12,13,46]. Recent evidence shows that, in populations under intense sexual conflict, quick directional environmental changes will alleviate male harm and overall sexual conflict, which may aid evolutionary rescue-. Given the observed quick local adaptation of male harm in response to directional thermal selection, our results show that these effects are likely to be transient in the case of global warming, at least in *Drosophila melanogaster* populations. This, however, would depend on the pace, extend and magnitude of male harm effects promoting evolutionary rescue vs. local adaptation.

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 plastic in lines adapted to cold temperatures (16-24ºC). In contrast, seminal fluid proteins (SFPs), responsible for copulatory harm[11,15,35] in this species, characterized the evolution of male seminal proteomes at warm (24-32ºC) vs. cold or moderate temperatures (20-28ºC) (Figs. 3-5). Our results thus show rapid divergent evolutionary responses of behavioural traits and male SFPs involved in pre-copulatory vs. copulatory male harm in response to temperature. 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[49]. Recent theoretical and empirical developments place metabolism as a causative nexus in the evolutionary interplay between ecology, life history, and sexual selection[31,49–51]. 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[52]. Thus, cold temperatures may place a general constraint on 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[29].

In contrast, there is ample evidence that hot temperatures have particularly strong effects on proteins and sperm phenotype and function across animals[53–57]. For example, high temperatures lead to a reduction in sperm production, motility, viability and longevity,

consequently affecting reproductive outcomes[54]. Moreover, although scarce and indirect, recent findings suggest that some of these effects may be mediated by temperature effects on seminal fluid proteins[58,59]. In particular, high temperatures increase entropy, lessening protein (un)folding and thus reducing the fraction of functional proteins[53]. 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 adaptation to hot temperature and, in a recent study with flies from the wild ancestral population, we show that hot temperature (28ºC) compromises SFPs effects on female receptivity, an important component of male copulatory harm to females[29]. 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 adapt quickly to recover the original levels of male harm to females. To conclude, our results show that adaptive responses to coarse-grain but natural temperature fluctuations can drive the divergent evolution of male harm mechanisms, and we suggest that these responses may be widespread across the tree of life. If so, inter- and intraspecific variation in temperature could help explain the extraordinary diversity of male harm adaptations across taxa and could potentially foster incipient speciation by contributing to establish reproductive barriers among populations.

Third, quick divergent local adaptation of pre- and post-copulatory mechanisms of harm would only be possible via strong selection operating on high levels of standing genetic variation in the ancestral population[60,61]. 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[62]. 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[29]. 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 adapted to 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), explaining the adaptability of our experimental populations. However, as discussed above plastic SFPs responses to hot temperatures in the ancestral population appeared maladaptive[29]. 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. 6). 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[63–68]. 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"[69]). 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*[65,70]. 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

In this study, we show evidence of quick local adaptation of male harm to females in response to temperature, which was characterized by divergent evolution of pre- vs. copulatory traits at cold vs. hot temperatures. 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. 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 we suggest a priority for future research should be to understand whether and how temperature may affect the evolution of female resistance to harm.

Methods

Fly populations and design experimental evolution

12 experimental populations, each with a controlled size of 100 males and 100 females, were established from the stock field collected population "Vegalibre" in 2020 (see Londoño-Nieto et al., 2023 for the details about the wild population). Populations 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. Four populations (replicates) evolved at each temperature regime (populations A, B, C and D evolved at 20±4°C -*cold*-; populations E, F, G and H at 24±4°C -*moderate*-, and populations I, J, K and L at 28±4°C -*hot*-). Populations were maintained in non-overlapping generations, precisely to control the population size per generation. Each generation begun by releasing 100 randomly selected same-aged males and females (N = 200) into a glass (16.5 x 19.5cm) population cage with two 75ml maize-malt medium bottles (7g of agar, 72 g of malt, 72g of maize, 9g of soya, 15g of live yeast, 20g of molasses, and 33ml of Nipagin mix –3g of methyl 4-hydroxy-benzoate and 28ml of ethanol 90%– per 1000ml of water). After setting up the population, we allowed them 6 days to interact, whereby eggs were collected on 6th day. Larvae were raised at a standardized density[71] on bottles with 75ml maize-malt medium. Virgins collected from these bottles were separated into same-sex vials and used to setup the next generation at 3-4d old. Note that we actively selected for early reproduction, which as stated above means that cumulative harm effects over time are not considered and thus selection for female resistance should be minimised[72,73]. Populations were assayed after 29 (for male harm experiments) or 30 (for proteomics experiments) generations of experimental evolution and two generations in which

all populations were reared under standardized conditions at 24±4°C, in order to erase parental and grand-parental effects. Evolution period started in February 2020 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. Thus, 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)

We collected experimental flies as virgins (i.e., within 6h of eclosion) under ice anesthesia into same-sex vials of 15 individuals and then randomly allocated them to either of the three temperature treatments: 20, 24, and 28°C, 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 maize-malt medium supplemented with live yeast, after which we immediately added one (monogamy; low sexual conflict) or three (polygamy; high sexual conflict) experimental males from the corresponding population to each female vial. We then observed flies 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)[41,74]. To estimate female reproductive success, we transferred flies to fresh vials twice a week. We incubated the vials containing focal female eggs 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. The differences in incubation time are due to differences in temperature during the first 3-4 days of each vial. We discarded and replaced males with young (2-4d old) virgin males (receiving the same treatment as described

above for original males) 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 (including the four replicates) for female reproductive success and survivorship were: (a) cold regime of evolution: at 20°C, $n_{polyandry}$ = 171 and $n_{monogamy}$ = 166, at 24°C: $n_{polyandry}$ = 163 and $n_{monogamy}$ = 162, and at 28°C: $n_{polyandry}$ = 163 and $n_{monogamy}$ = 165, (b) moderate regime of evolution: at 20°C, $n_{polyandry}$ = 167 and $n_{monogamy}$ = 168, at 24°C: $n_{polyandry}$ = 165 and $n_{monogamy}$ = 162, and at 28°C: $n_{polyandry}$ = 173 and $n_{monogamy}$ = 172 and (c) hot regime of evolution: at 20°C, $n_{polyandry}$ = 167 and $n_{monogamy}$ = 157, at 24°C: $n_{polyandry}$ = 152 and $n_{monogamy}$ = 153, and at 28°C: $n_{polyandry}$ = 169 and $n_{monogamy}$ = 165. For behavioural observations we used 120 vials per treatment (30 per replicate). We modeled 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*[75] and *glmmTMB*[76] packages in RStudio (version 4.2.2). We modeled survivorship as the response variable in a Cox proportional hazard model with the same fixed and random effects using *coxme* and *survminer* packages[77,78]. For courtship and aggression rates we used zero inflated distribution and for female rejection rate we used negative binomial distribution. As our replicates are different populations, we also fitted random slopes models for correlated fixed effects of temperature evolution regime and temperature treatment[79]. 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[80]; 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 explore specifically if sexual conflict responds differentially to adaptive temperatures, we run extra models using the data only from cold and hot experimental evolution regimes due to temperatures included inside the moderate regime range coincide with the temperatures evaluated as treatments (i.e., moderate regime does not include maladaptive temperatures). We modeled experimental evolution regime, mating system and an extra factor that we called adaptive temperature with two levels (yes/ no) and the interaction between mating system and adaptive temperature as fixed effects, and replicate population as random effect. In all cases where 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)

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. We aspirated the mated/virgin males into cryovials before flash-freezing them. 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[37,38,43]. 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 (A to L) 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 populations A, B, C and D evolved in 20±4°C; 24 from populations E, F, G and H evolved in 24±4°C, and 24 from populations I, J, K and L evolved in 28±4°C). Our quantitative proteomics analysis was conducted in accordance with the sample preparation protocol SWATH-MS[81]. 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 analyze 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 analyze our data sets, using *glmnet* [82] 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[83] (Zou & Hastie, 2005). We also analyzed our data sets using tests of reduction of dimensionality PLS-DA, using *mixOmics*[84] 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[85]. Finally, we also identified all proteins described as seminal fluid proteins (SFPs), based on a high-confidence SFPs reference list from Sepil et al.[43] and Wigby et al.[86]. 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[87] in RStudio.

GxE assay (experiment 3)

We used 30 male genotypes (isolines) derived from wild-caught flies from the ancestral wild population from which stocks used in experiment 1 and 2 were derived ("Vegalibre" population). We established isolines through 10 generations of inbreeding, resulting in flies sharing at least 96% of their genome[88]. 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 (i.e., within 6h of eclosion) 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. *spa*^{pol} is a recessive phenotypic marker that can be used for paternity assignment. We collected *spa*^{pol} eggs directly from our Vegalibre *spa*^{pol} stock population, raised them at standard density, and collected virgin adults that we placed into same-sex vials until the beginning of the experiment. To begin the experiment, we placed *wt* males from each isoline in individual vials containing maize-malt 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°C, 24°C, 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). Flies remained at those temperatures until the end of assays. 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 of focal *wt* males daily, at which time dead spa^{pol} flies were replaced with flies of similar age from parallel stock vials collected every two weeks. To estimate the reproductive success of focal wt males we transferred flies to fresh vials twice a week, incubated vials with eggs and then froze them for subsequent counting of the F1 offspring. We calculated reproductive success of focal males as the proportion of sired offspring vs. offspring sired by rival males. We modelled reproductive success of the *wt* males as the response variable in a GLMM with temperature as a fixed effect and isoline and their interaction as random effects[89] using *glmmTMB*[76]. We used a Beta regression model calculating reproductive success as a proportion of *wt* individuals within the total (*wt* + spa^{pol}) number of individuals[90]. We used Nakagawa's R-squared[91] to extract the variance explained by each model, analyzed random effects using *ranef* function from lme4[75], and tested via likelihood ratio tests for significance.

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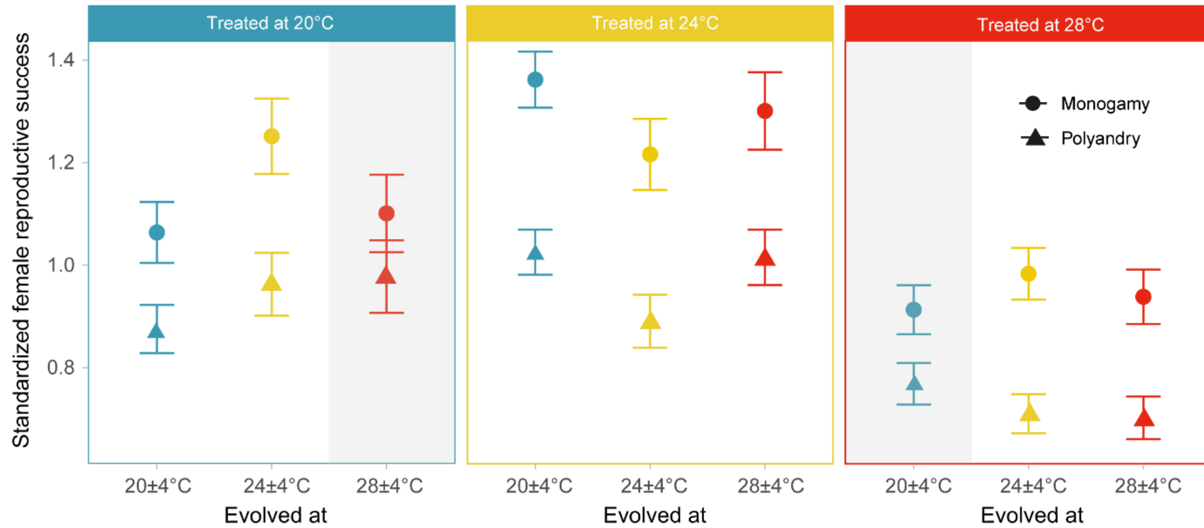

Figure 1 | Effect of mating system, temperature treatment and experimental evolution regime on female fitness. Female reproductive success (mean ± s.e. of four replicates) across mating systems (monogamy and polyandry), temperature treatments (20,24 and 28°C) and experimental evolution thermal regimes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

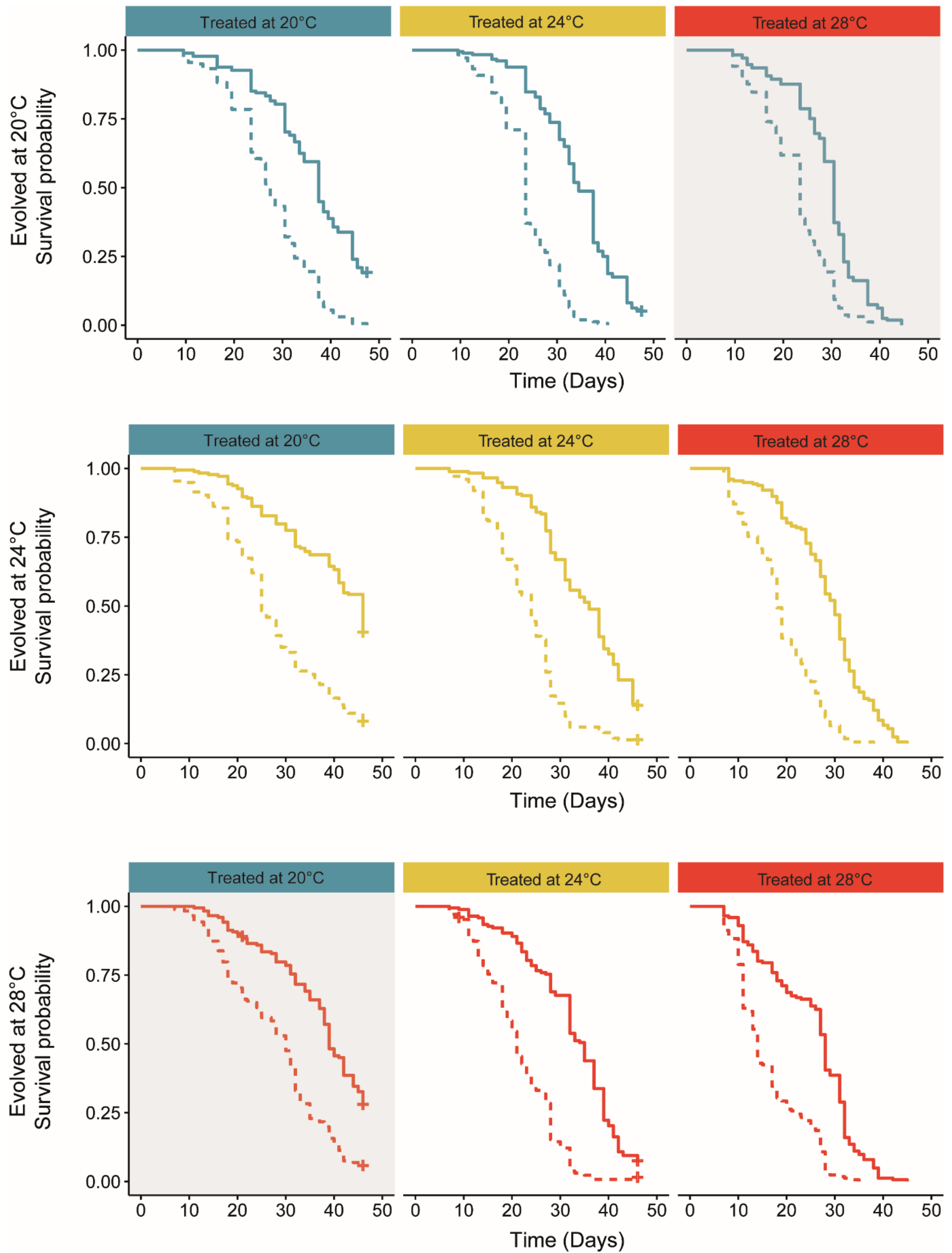

Figure 2 | Effect of mating system, temperature treatment and experimental evolution regime on female survival. Female survival (four replicates) across mating systems, temperature treatments and experimental evolution thermal regimes. The difference between female survival kept in monogamy (solid lines) 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).

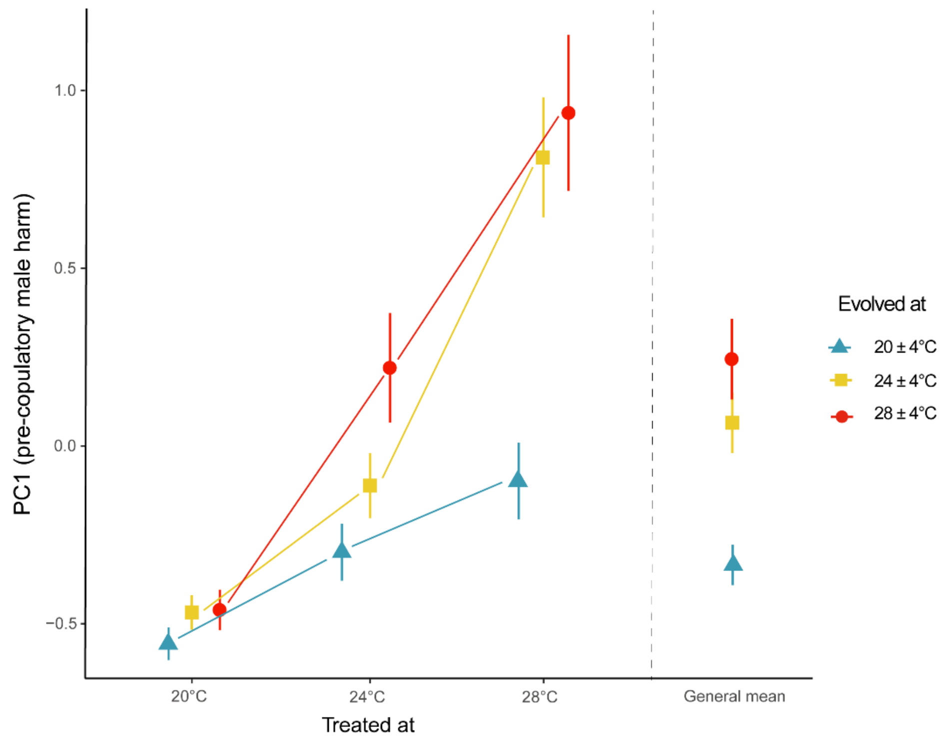

Figure 3 | Effect of temperature treatment and experimental evolution regime on pre-copulatory male harm. Frequency patterns (mean ± s.e.) for the PC1 from a PCA in which all behaviours involved in pre-copulatory harm (courtship intensity, female rejection and male-male aggression) were examined together for increased conflict (i.e., polyandry). We took this PC1 as an overall index of male harassment to females.

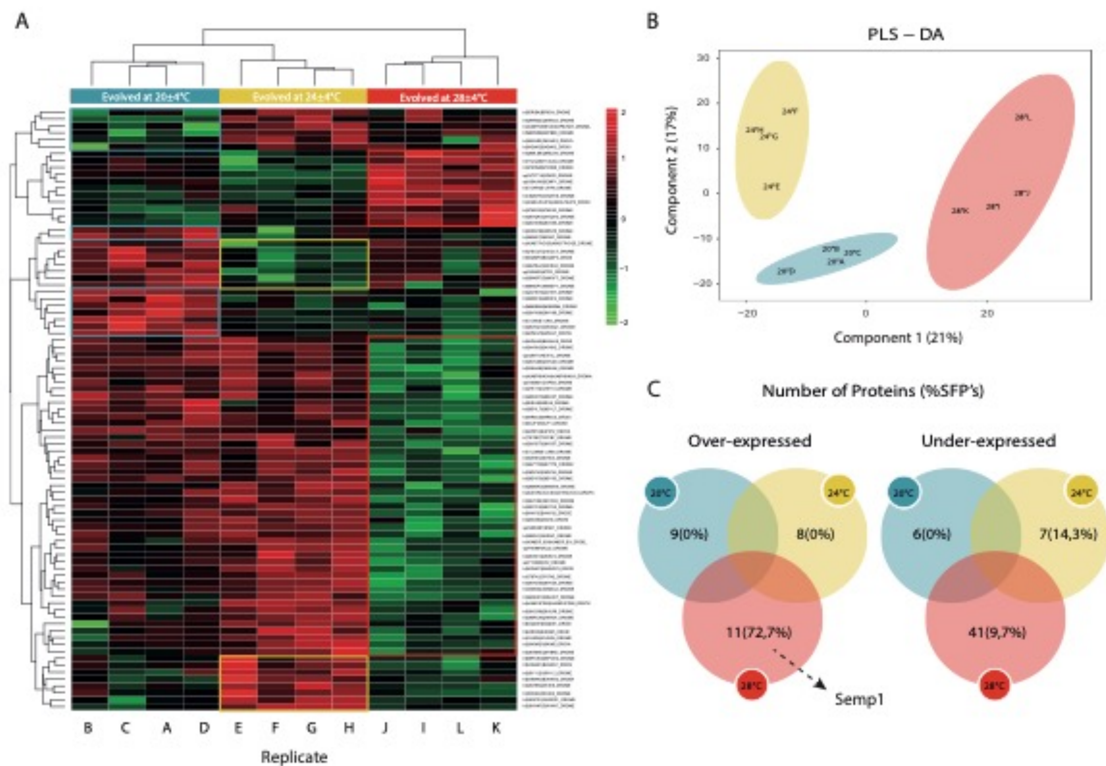

Figure 4 | Effect of experimental evolution regime on virgin male's seminal proteome production. A) Heatmap showing the abundance of 87 proteins selected by the Elastic net regression. Each cell gives the across-biological replicate mean for that protein in each experimental evolution thermal regime 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.

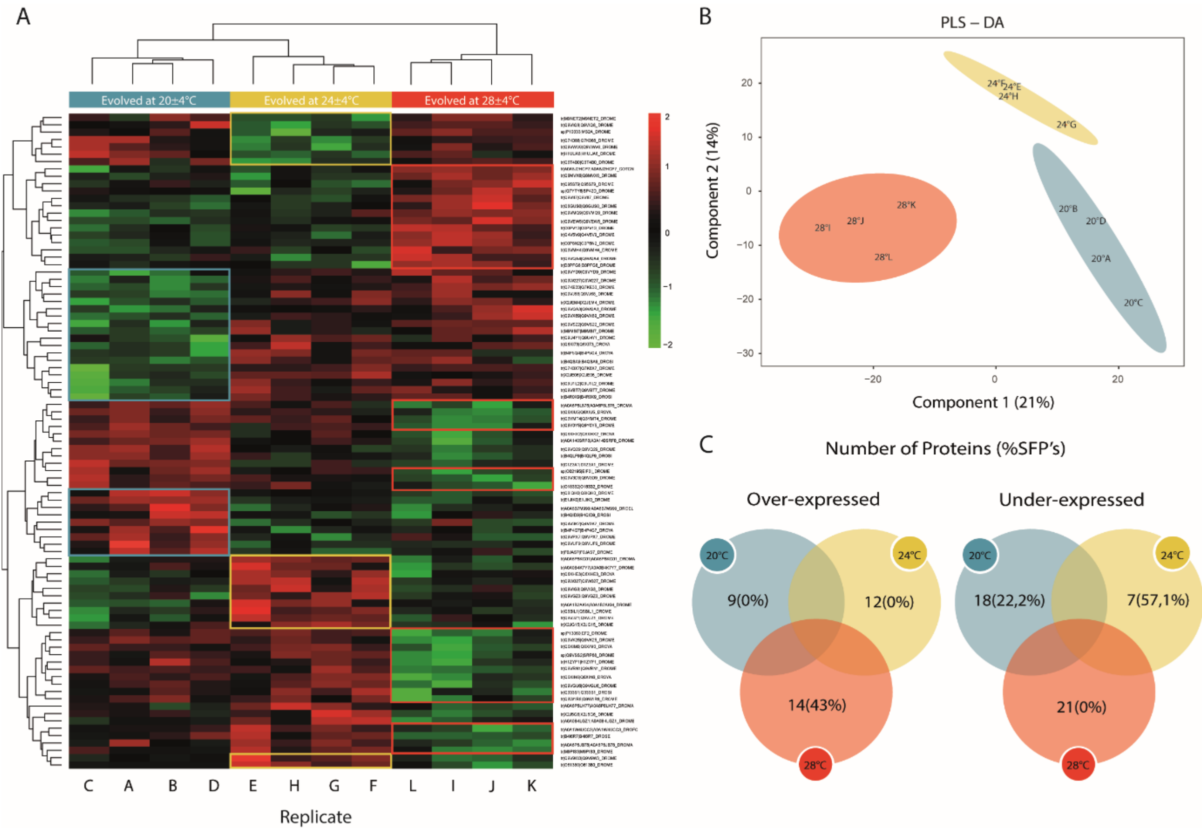

Figure 5 | Effect of experimental evolution regime on mated male's seminal proteome production. A) Heatmap showing the abundance of 89 proteins selected by the Elastic net regression. Each cell gives the across-biological replicate mean for that protein in a given experimental evolution thermal regime 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.

Supplementary Information

Materials and Methods

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 13000rpm 15 min. Supernatants were discarded and pellets suspended in 50µL of Laemmli buffer 1.5X. 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 (7.5µg/sample to SWATH and 25µg 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 (25ug). 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 37 ºC on. Trypsin digestion was stopped with 10% TFA, the SN was removed, and the library gel slides were dehydrated with pure ACN[1]. 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 0.5mm; Eksigent) and desalted with 0.1% TFA at 5 µl/min during 5 min. Peptides were then 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). 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 uL 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 analyzing 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[2] 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 actually 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 7.5µg 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[1]. 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 300nl/min. Peptides were analysed in a mass spectrometer nanoESI qQTOF (6600plus TripleTOF, ABSCIEX).

Sample was ionized in a Source Type: Optiflow < 1 uL 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.

Supplementary references

1. Shevchenko, A. *et al.* Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 14440–14445 (1996).
2. Shilov, I. V. *et al.* The paragon algorithm, a next generation search engine that uses sequence temperature values sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. *Molecular and Cellular Proteomics* **6**, 1638–1655 (2007).

Results

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. S2-5). Courtship rate and rejection rate per courtship exhibited a trend in the same direction, but the effect was not significant (Figs. S6-9), 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 lines (i.e., flatter reaction norms: experimental evolution x temperature treatment interaction: X^2_2 = 11.81, P = 0.018; Figs. S2-3 and Table S4). Courtship intensity exhibited a clear trend in the same direction as aggression rate (Figs. S8-9) 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. S4-7 and Table S4a-d). Finally, courtship rate varied greatly across mating systems and the strength of this effect varied considerably across temperature treatments (mating system * treatment temperature interaction:X^2_2 = 18.37, P < 0.001; Figs. S8-9), suggesting less harassment in flies treated at 20°C and more in flies treated at 28°C (Figs. S8-9; Table S6).

Table S1. 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.

a)

Experimental evolution regime	***LRS***				
	Effect	*F*	*Df*	*Df.res*	*P*
20±4°C	Temperature tratment	28.37	2	983.24	**<0.001**
	Mating system	33.08	1	983.03	**<0.001**
24±4°C*	Temperature tratment	0.96	2	1.99	0.510
	Mating system	40.51	1	994.59	**<0.001**
28±4°C	Temperature tratment	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	***LRS***				
	Estimate	*SE*	*df*	*t.ratio*	*P vlaue*
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	***LRS***					
	Contrast	*Estimate*	*SE*	*df*	*t.ratio*	*P vlaue*
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**

Table S2. 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.

a)

Experimental evolution regime	***Lifespan***			
	Effect	*Chisq*	*Df*	*P*
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*	*t.ratio*	*P vlaue*
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*
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*	*t.ratio*	*P vlaue*
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**

Table S3. 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***
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.ratio*	*P vlaue*
20±4°C	20° – 24°	-0.31	0.12	1039	-2.64	**0.022**
	28° – 24°	0.19	0.12	1039	1.62	0.237
	28° – 20°	0.50	0.12	1039	4.30	**<0.001**
24±4°C	20° – 24°	-0.32	0.12	1039	-2.74	**0.017**
	28° – 24°	0.62	0.12	1039	5.29	**<0.001**
	28° – 20°	0.94	0.12	1039	8.03	**<0.001**
28±4°C	20° – 24°	-0.47	0.12	1039	-4.00	**<0.001**
	28° – 24°	0.47	0.12	1039	4.09	**<0.001**
	28° – 20°	0.94	0.12	1039	8.03	**<0.001**

Table S4. 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*
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*	*t.ratio*	*P vlaue*
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 S5. 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.

a)

Experimental evolution regime	***Rejection rate***			
	Effect	*Chisq*	*Df*	*P*
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*	*t.ratio*	*P vlaue*
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*
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**

d)

Experimental evolution regime	***Rejection rate per courtship***					
	Contrast	*Estimate*	*SE*	*df*	*t.ratio*	*P vlaue*
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**

Table S6. 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.

a)

Temperature treatment	***Courtship rate***			
	Effect	*Chisq*	*Df*	*P*
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*	*t.ratio*	*P vlaue*
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**

Table S7. 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**
C0PV13						+		Sexual reproduction
E1JHF8					+			Sexual reproduction
P10333				–			Identical protein binding	Mating, positive regulation of octopamine signaling pathway, positive regulation of ovulation, sexual reproduction, sperm competition
Q4V6H2					–		Peroxidase activity, thioredoxin-dependent peroxiredoxin activity	Response to oxidative stress, sexual reproduction
Q6GUS0						+		Sexual reproduction
Q7K088				–			Odorant binding	Sensory perception of smell, sensory perception of chemical stimulus, sexual reproduction
Q7K110					–		Transferase activity	Protein N-linked glycosylation, encapsulation of foreign target
Q7K5N8					+		Cysteine-type endopeptidase activity, cysteine-type peptidase activity	Proteolysis, proteolysis involved in protein catabolic process, 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 signaling 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.
Q8MLS8					+			Proteolysis, sexual reproduction
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 binding, zinc ion binding	Peptide catabolic process, proteolysis, sexual reproduction
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	Defense 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.
Q9VQA3		–			+			Proteolysis, sexual reproduction
Q9VWV6				–			Iron ion binding	Iron ion transmembrane transport, iron ion transport, olfactory behavior, 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.

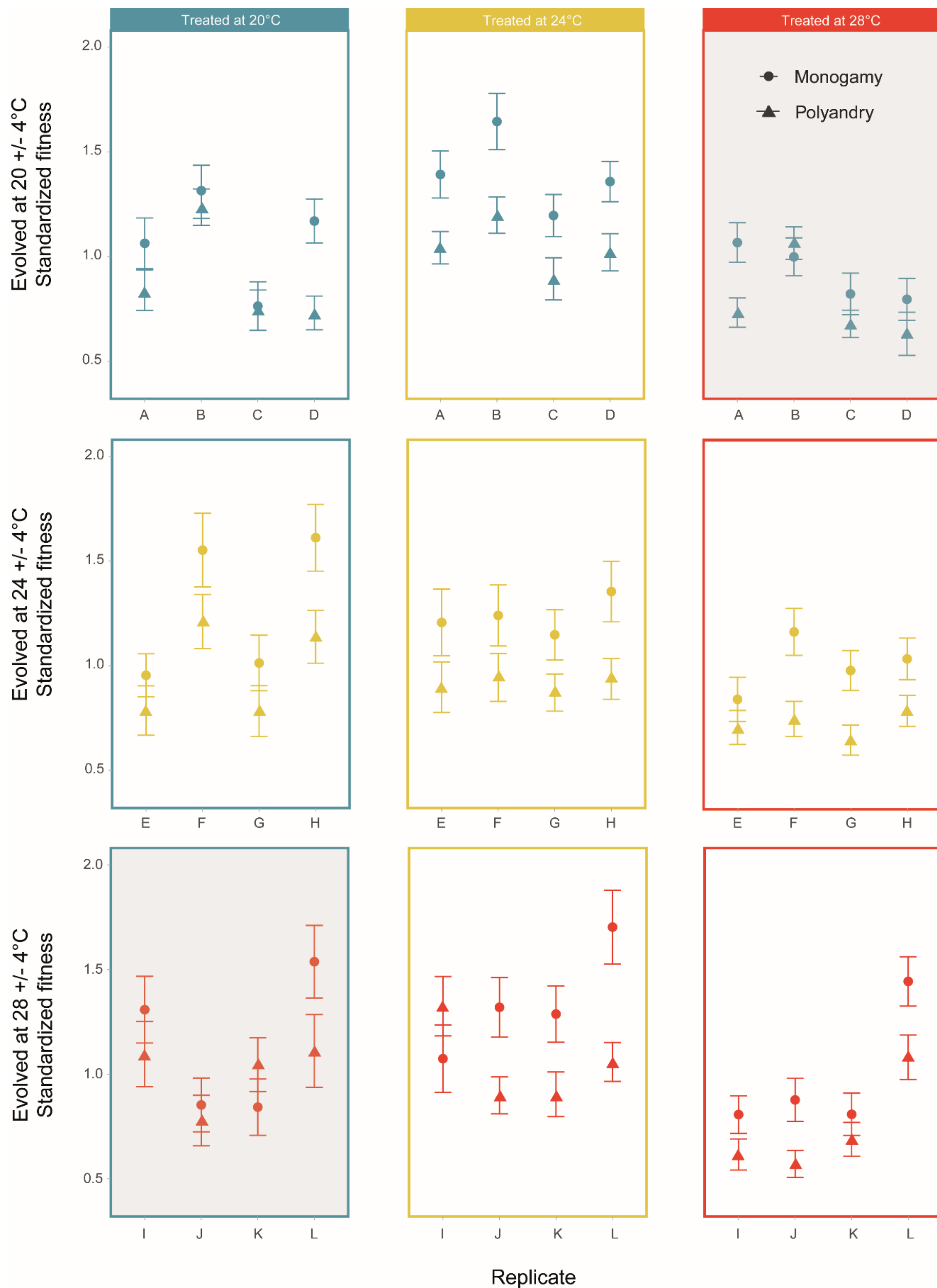

Figure S1 | Effect of mating system, temperature treatment and experimental evolution regime on female fitness by replicate. Female reproductive success (mean ± s.e.) across treatments and replicates. Data were standardized for each experimental evolution line. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective line.

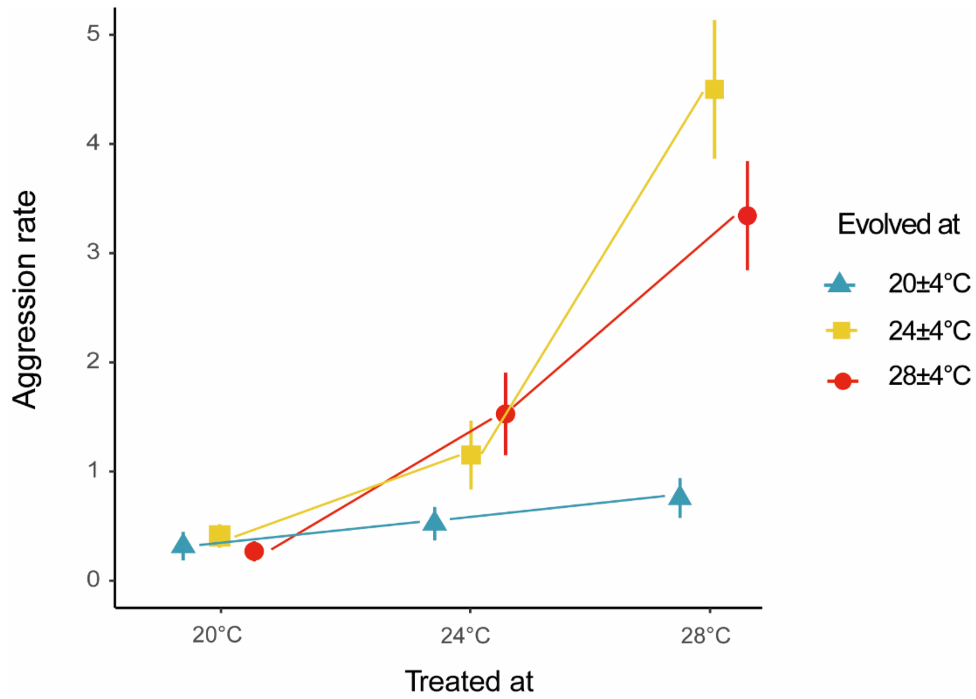

Figure S2 | Effect of temperature treatment and experimental evolution regime on male-male aggression rate. Aggressions male-male per hour (mean ± s.e.; four replicates) across temperature treatments and experimental evolution thermal regimes.

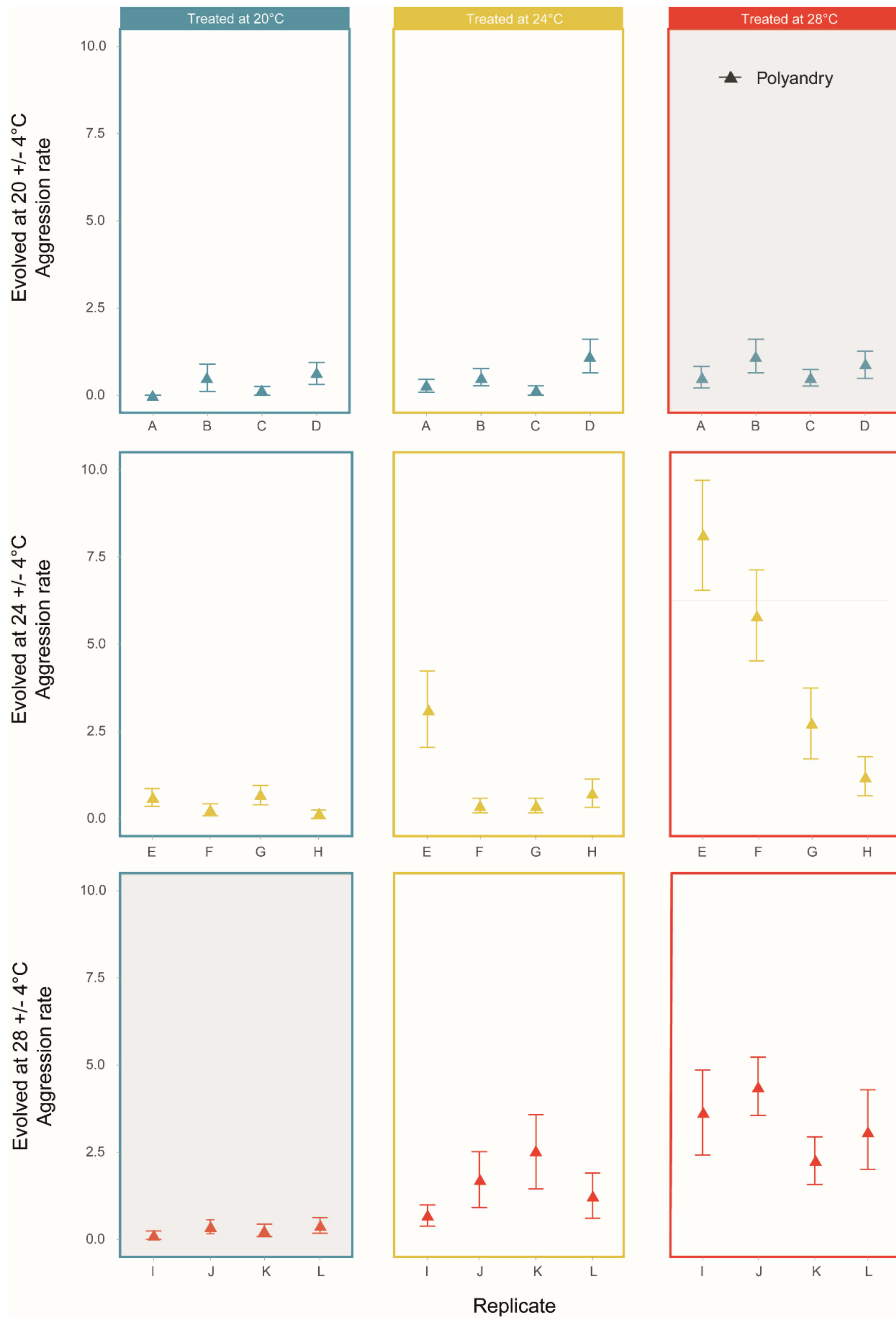

Figure S3 | Effect of temperature treatment and experimental evolution regime on male-male aggression rate by replicate. Aggressions male-male per hour (mean ± s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective line.

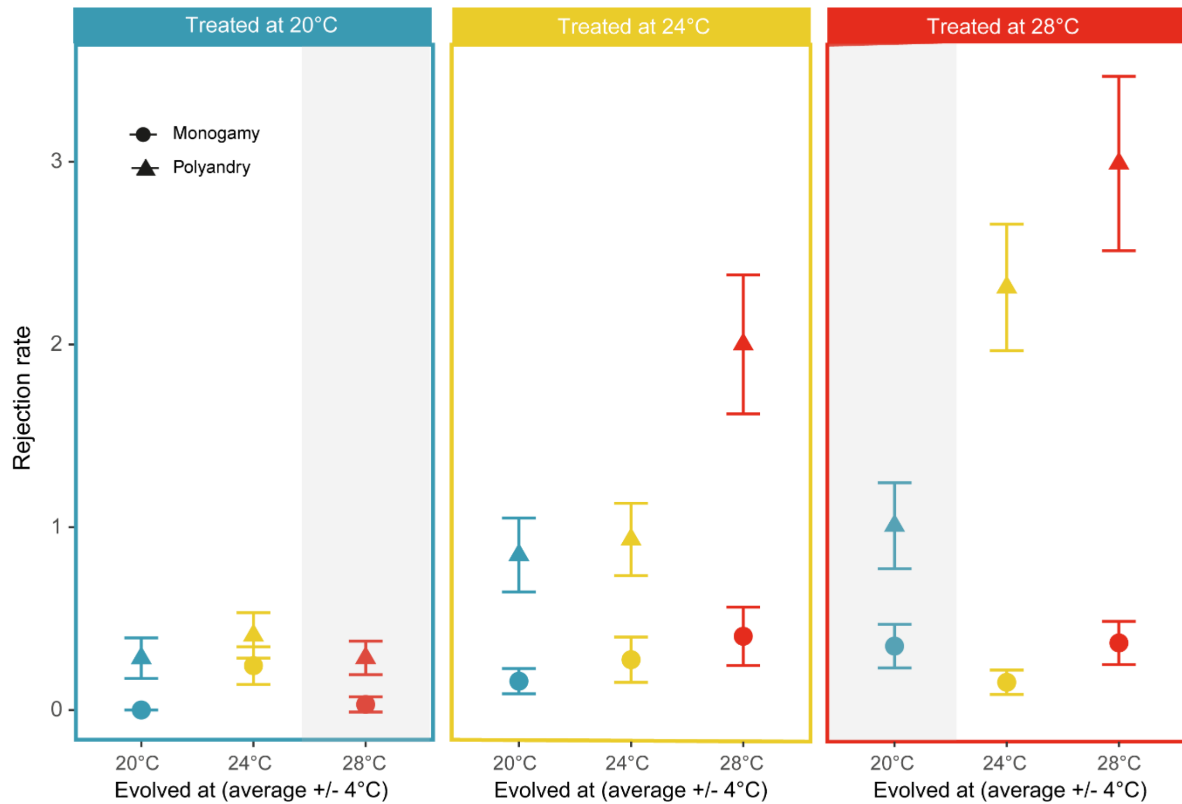

Figure S4 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate. Female rejections per hour (mean ± s.e.; four replicates) across temperature, mating system treatments and experimental evolution thermal regimes. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective line.

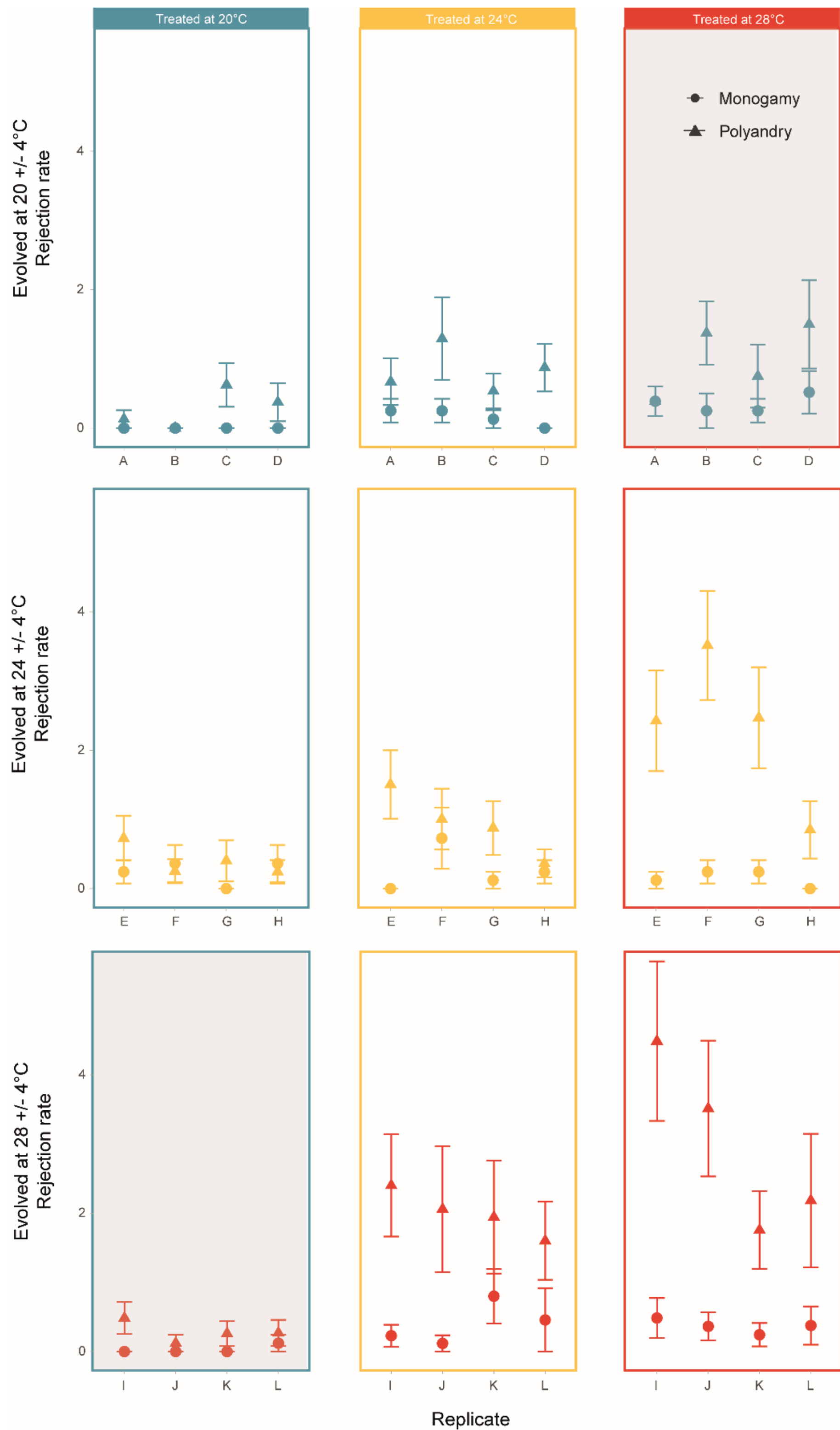

Figure S5 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate by replicate. Female rejections per hour (mean ± s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective line.

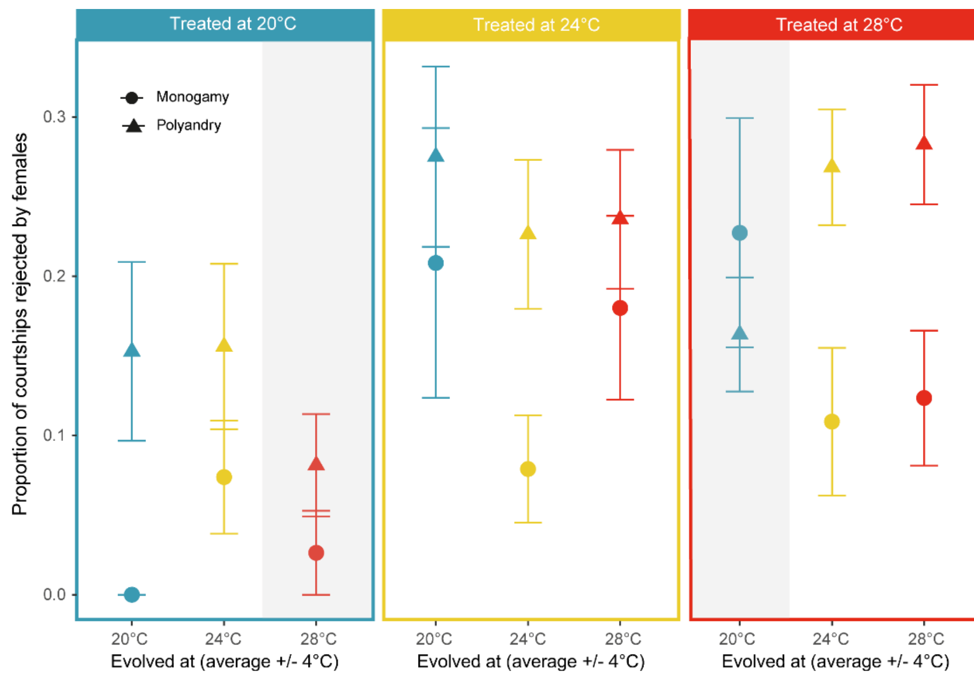

Figure S6 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate per courtship. Proportion of courtships rejected by females (mean ± s.e.; four replicates) across temperature, mating system treatments and experimental evolution thermal regimes. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective line. Rejection rate per courtship was only calculated 30% of the observation time (when courtship rate was different from 0).

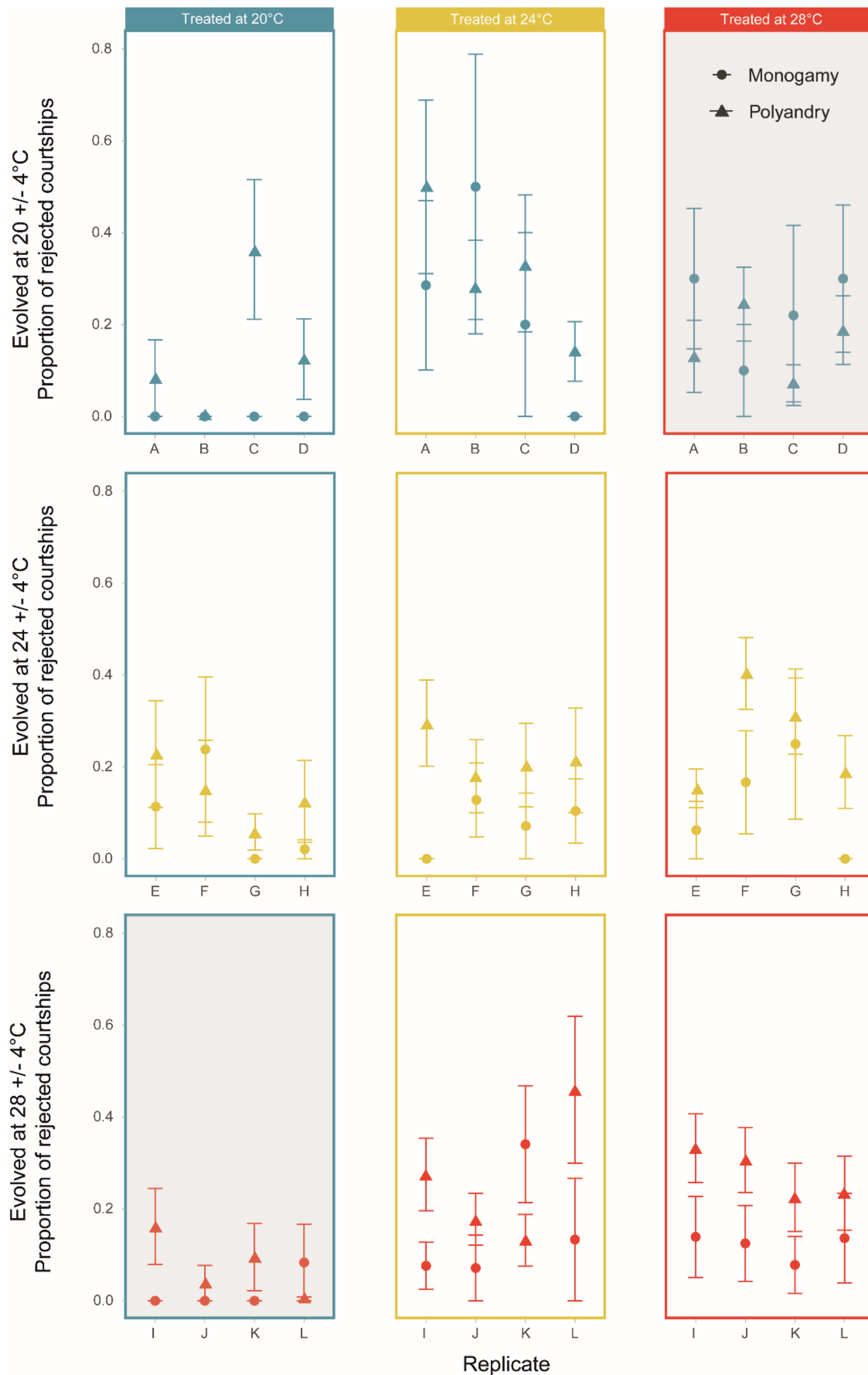

Figure S7 | Effect of mating system, temperature treatment and experimental evolution regime on female rejection rate per courtship by replicate. Proportion of courtships rejected by females (mean ± s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective line. Rejection rate per courtship was only calculated 30% of the observation time (when courtship rate was different from 0).

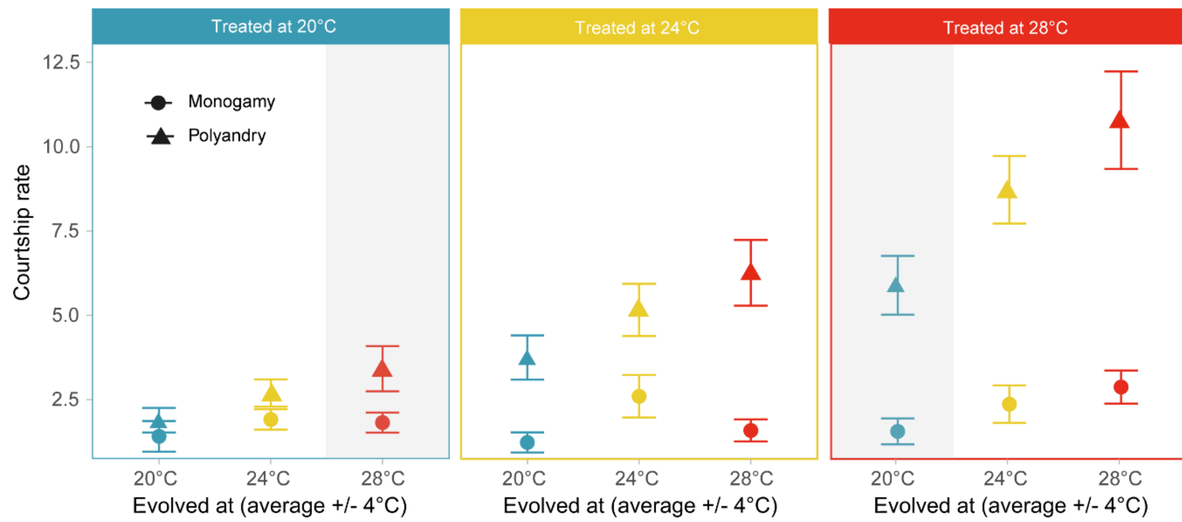

Figure S8 | Effect of mating system, temperature treatment and experimental evolution regime on courtship rate. Courtship per female per hour (mean ± s.e.; four replicates) across temperature, mating system treatments and experimental evolution thermal regimes. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective line.

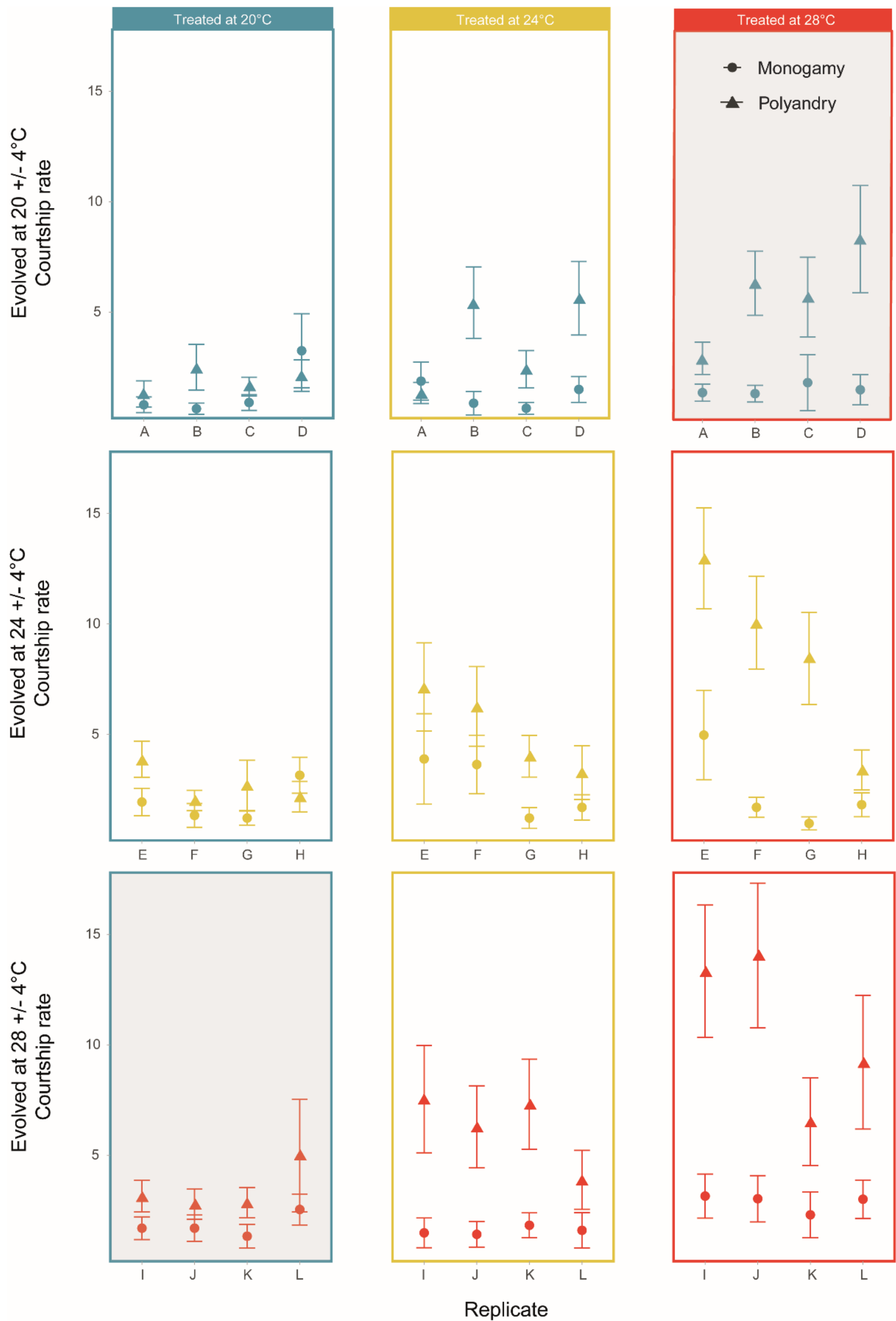

Figure S9 | Effect of mating system, temperature treatment and experimental evolution regime on courtship rate by replicate. Courtship per female per hour (mean ± s.e.) across treatments and replicates. Shaded panels denoted temperature treatments outside the experimental evolution thermal regime for the respective line.

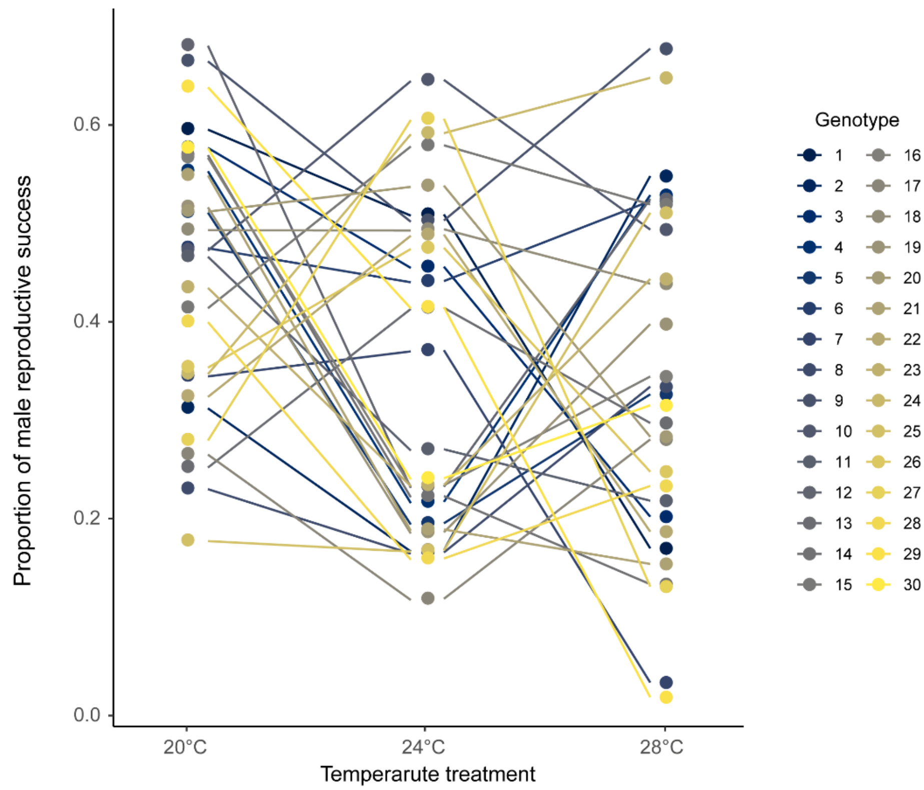

Figure 6 | Genotype-by-environment interactions for male reproductive success. Reaction norms for male reproductive success in 30 genotypes analyzed across three temperature treatments.