Thermal phenotypic plasticity of pre- and post-copulatory male harm buffers sexual conflict in wild *Drosophila melanogaster*

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ABSTRACT

Strong sexual selection frequently leads to sexual conflict and ensuing male harm, whereby males increase their reproductive success at the expense of harming females. Male harm is a widespread evolutionary phenomenon with a strong bearing on population viability. Thus, understanding how it unfolds in the wild is a current priority. Here, we sampled a wild *Drosophila melanogaster* population and studied male harm across the normal range of temperatures under which it reproduces optimally in nature by comparing female lifetime reproductive success and underlying male harm mechanisms under monogamy (i.e., low male competition/harm) vs. polyandry (i.e., high male competition/harm). While females had equal lifetime reproductive success across temperatures under monogamy, polygamy resulted in a maximum decrease of female fitness at 24°C (relative harm; H = 0.36), reducing its impact at both 20°C (H = 0.22), and 28°C (H = 0.10). Furthermore, female fitness components and pre- (i.e., harassment) and post-copulatory (i.e., ejaculate "toxicity") mechanisms of male harm were asymmetrically affected by temperature. At 20ºC, male harassment of females was reduced, and high male competition accelerated female actuarial senescence. In contrast, at 28ºC male ejaculate effects on female reproduction were modulated, and high male competition mostly resulted in accelerated reproductive ageing. We thus show that, across a natural thermal range, sexual conflict processes and their effects on female fitness components are plastic and complex. As a result, the net effect of male harm on overall population viability is likely to be lower than previously surmised. We discuss how such plasticity may affect selection, adaptation and, ultimately, evolutionary rescue under a warming climate.

INTRODUCTION

Females and males share the common goal of siring offspring. This central tenet of sexual reproduction enforces a certain degree of cooperation between the sexes. However, product of anisogamy, general asymmetries in the evolutionary interests around reproduction across the sexes lead to distinct sex roles and thus diverging intensity and form of sexual selection (Arnqvist & Rowe, 2005; Chapman, Arnqvist, Bangham, & Rowe, 2003). This phenomenon, termed sexual conflict, favours traits in one sex that might be costly for the other (Parker, 1979), and can thus lead to antagonistic male-female coevolution (Arnqvist & Rowe, 2005). Sexually antagonistic co-evolution has received much attention, and is recognized as a fundamental process in evolution due to its role in shaping male and female adaptations (Bonduriansky, Maklakov, Zajitschek, & Brooks, 2008), in contributing to drive reproductive isolation and speciation (Arnqvist, Edvardsson, Friberg, & Nilsson, 2000; Bonduriansky, 2011; Bonduriansky & Chenoweth, 2009; Gavrilets, 2014), and as a major determinant of population demography (Kokko & Brooks, 2003; Bonduriansky & Chenoweth, 2009; Berger et al., 2016). Specifically, sexual conflict has been shown to have profound consequences for female fitness and population growth when it favours male reproductive traits that increase male intra-sexual competitive ability at the expense of harming females (i.e., male harm, Crudgington & Siva-Jothy, 2000; Wigby & Chapman, 2004).

Harmful male adaptations are widespread and incredibly diverse and sophisticated across the tree of life (Arnqvist & Rowe, 2005). For example, male harassment of females during pre-copulatory competition for mating has been documented in myriad vertebrate and invertebrate species (García-Roa et al., 2021), driving antagonistic male-female co-evolution in a host of behavioural and morphological traits (Arnqvist & Rowe, 2005). Male harm adaptations in the context of post-copulatory competition are similarly widespread in invertebrates, featuring (amongst others) toxic ejaculates (Wigby & Chapman, 2005), love darts (Koene & Schulenburg, 2005), and a range of male adaptations for traumatic insemination that range from genital ablation to spiny penises (Crudgington & Siva-Jothy, 2000; Lange, Reinhardt, Michiels, & Anthes, 2013). Importantly, beyond driving male-female phenotypes and associated diversification processes, male harm generally leads to a “reproductive tragedy of the commons” that can substantially impact population demography by depressing net female productivity (Arnqvist & Tuda, 2010; Berger et al., 2016; Holland & Rice, 1999; Rankin et al., 2011), and even facilitate population extinction (Le Galliard, Fitze, Ferriere, & Clobert, 2005). Unsurprisingly, understanding what factors underlie male

harm evolution, its diversity in form, strength, and outcomes, is a main concern in evolutionary biology.

Despite a growing number of studies in the field of sexual conflict, most have been conducted under uniform laboratory conditions, frequently in populations adapted to stable environments for thousands of generations (Chapman et al., 2003b; Hopkins et al., 2020; Wigby & Chapman, 2004, 2005). In contrast, recent research has highlighted the role of ecology in shaping the evolution of traits under sexual conflict (Arbuthnott et al., 2014; García-Roa et al., 2019; MacPherson et al., 2018; Perry & Rowe, 2018; Yun et al., 2017), including habitat complexity (Malek & Long, 2019; Miller & Svensson, 2014; Myhre, Forsgren, & Amundsen, 2013), nutritional status (Fricke, Bretman, & Chapman, 2010), or sex ratio and population density (Chapman et al., 2003a). For example, Gomez-Llano and colleagues (2018) recently showed that conspecific densities and the presence of heterospecifics modify the intensity and outcome of sexual conflict in the banded demoiselle (*Calopteryx splendens*), and the spatial complexity of the environment in which mate competition occurs mediates how sexual conflict operates in fruit flies (Yun et al., 2018). The incorporation of more realistic ecological scenarios in the study of sexual conflict is thus a key avenue to disentangle the evolution of male harm, and its consequences for population viability (Cornwallis & Uller, 2010; Fricke, Perry, Chapman, & Rowe, 2009; Plesnar-Bielak & Łukasiewicz, 2021).

Temperature is recognized as a crucial abiotic ecological factor due to its impact on life history traits and physiological and behavioural responses (De Lisle, Goedert, Reedy, & Svensson, 2018; Kim, Jang, & Lee, 2020; Miler, Stec, & Czarnoleski, 2020; Monteiro et al., 2017). Furthermore, in nature temperature varies widely both within and across spatio-temporal scales (e.g., daily, inter-seasonal and intra-seasonal variation), and may consequently have short, medium, and long-term effects on organism phenotypes that can impact many different aspects of its reproductive behaviour (e.g., sex-specific potential reproductive rates, operational sex ratios, density, etc.; García-Roa et al., 2020). In fact, a recent meta-analysis suggests that temperature may have a sizeable effect on sexual selection processes even when fluctuations occur well within the normal range of temperature variation for the studied species (García-Roa et al., 2020). This latter finding is particularly relevant given that we know almost nothing about how average temperature fluctuations, such as those experienced by wild populations during their reproductive season, affect male harm and sexual selection at large.

Our aim was to contribute to fill this gap in knowledge by studying how male harm responds to temperature variation that mimics average fluctuations that are normal during the reproductive season, using *Drosophila melanogaster* flies sampled from a wild population. *D. melanogaster* is an ideal subject for this study for two main reasons. First, it exhibits high levels of male- male competition and well characterized pre- and post-copulatory male harm mechanisms, and is perhaps the main model species in the study of sexual conflict (Arbuthnott et al., 2014; Chapman et al., 2003a; MacPherson et al., 2018; Malek & Long, 2019; Wigby & Chapman, 2005). During male-male pre-copulatory competition over access to mating, males harm females via intense harassment that causes physical injuries, interferes with female behaviours such as egg-laying or feeding, and results in energetically costly resistance to males (Bretman & Fricke, 2019; Partridge & Fowler, 1990; Teseo, Veerus, Moreno, & Mery, 2016). In the context of sperm competition over fertilizations, males can also harm females via “toxic” ejaculates, whereby certain male seminal fluid proteins manipulate female re-mating and egg-laying rates to the male advantage, but at a costs to females in terms of lifespan and lifetime reproductive success (Chapman et al., 2003b; Sirot et al., 2009; Wigby & Chapman, 2005). These proteins are secreted by male accessory glands, have been well characterised, and are strategically allocated by males in response to variation in the socio-sexual context (Hopkins et al., 2019; Sirot, Wolfner, & Wigby, 2011). Second, despite the fact that almost all work on male harm has, to date, been conducted in laboratory strains adapted to stable temperature conditions, *D. melanogaster* reproduces in the wild under a wide range of temperatures (Dukas, 2020; Kapun et al., 2018).

We collected flies from a continental wild population in Requena (Spain) that experiences significant fluctuations in temperature even during the mildest months when it is reproductively active (e.g., July: average: 24.9°C, average min: 19.8°C, average max: 30.1°C). After acclimation of the resulting population to the laboratory conditions under a fluctuating temperature regime mimicking natural conditions, we conducted a series of behavioural and fitness assays to gauge how temperature variation within a normal range affects: a) the overall impact of male-male competition on female lifetime reproductive success (i.e., male harm), b) how the net effects of harm are accrued in terms of different female fitness components (i.e., reproductive rate, actuarial ageing and reproductive ageing), and c) underlying male pre-copulatory (i.e., harassment) and post-copulatory (i.e., ejaculate “toxicity”) harm mechanisms.

RESULTS

Lifetime reproductive success and related fitness components

LRS- We detected a significant temperature x mating system interaction for female lifetime reproductive success (χ^2_1= 16.931; p < 0.001, Fig. 1), and a marginally non-significant temperature effect (χ^2_1= 3.712; p= 0.054). Running models separately for each temperature level, mating system affected lifetime reproductive success at 20°C (χ^2_1= 4.575; p= 0.034), and at 24°C (χ^2_1=12.569; p < 0.001), but not at 28°C (χ^2_1= 2.424; p= 0.122). The decrease in LRS in polyandry vs monogamy peaked at 24°C (H = 0.36) and was 1,6 (H = 0.22) and 3,4 times smaller (H = 0.10) at 20 and 28°C, respectively. Rate-sensitive fitness estimates show that estimated population costs are dependent on background growth rates (Fig. 2), and in general particularly accused in decreasing populations.

Early reproductive rate- We did not detect a significant temperature x mating system interaction (χ^2_2= 0.690; p= 0.7080), or a significant main mating system effect (χ^2_1= 3.287; p= 0.069), but as expected we did detect a significant main temperature effect (χ^2_2= 84.347; p < 0.001; Fig. SI1).

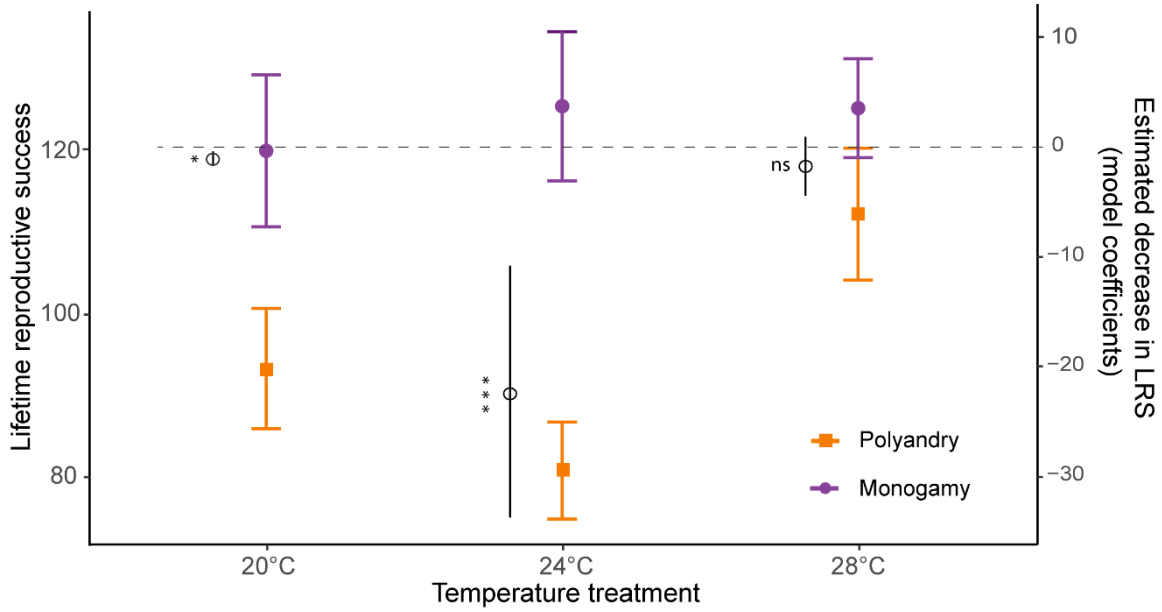

Figure 1. Female lifetime reproductive success (mean ± SEM) across temperature and mating system treatments. Second axis represents the model coefficient estimates (95% CI) for the estimated variation in lifetime reproductive success that ensue with polyandry vs. monogamy, across the different temperature treatments. Estimates are coefficients from GLMs fitted separately for the three temperature treatments.

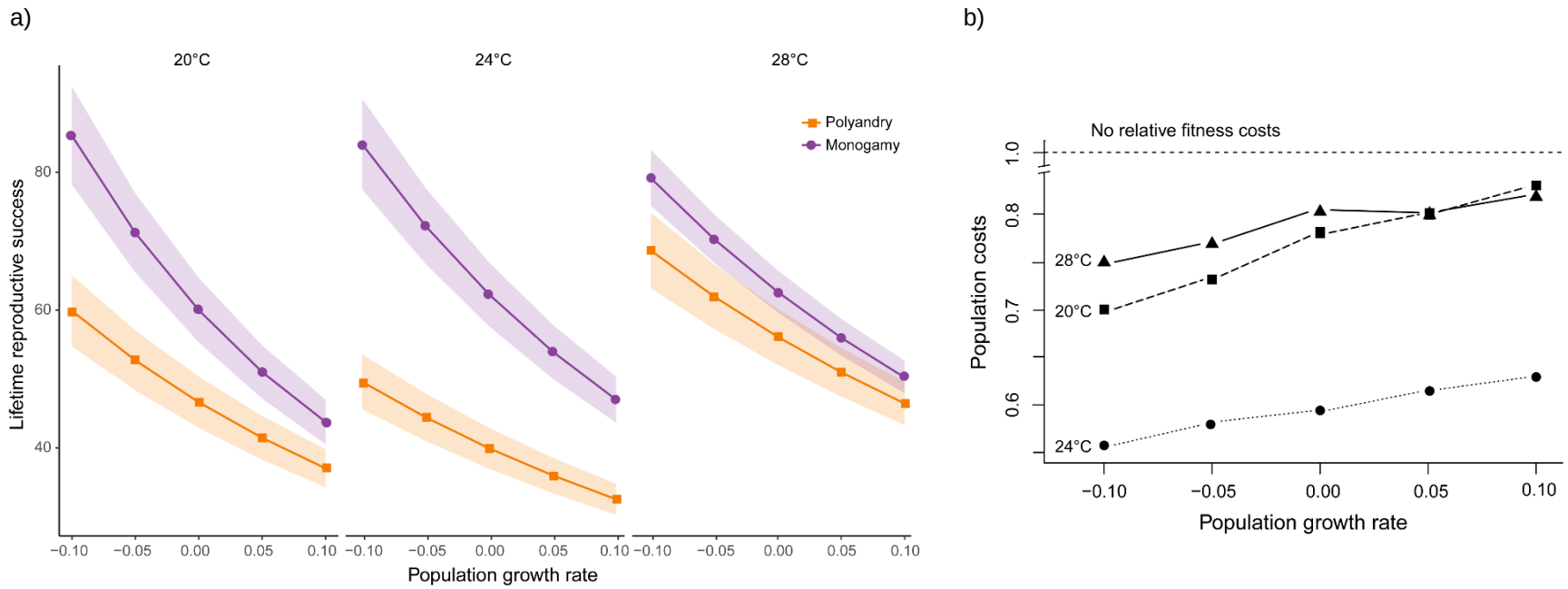

Figure 2. a) Lifetime reproductive success (mean ± SEM) for different population growth rates across temperature and mating system treatments, b) relative population fitness costs of polyandry vs. monogamy for different population growth rates for experimental temperatures. Rate-sensitive fitness estimates were modelled based on our LRS measures.

Reproductive senescence- We detected a marginally nonsignificant temperature x mating system effect for reproductive ageing (i.e., number of offspring during weeks 1 and 2 minus number of offspring during weeks 3 and 4) (χ^2_2= 5.599; p= 0.060; Fig. SI1), and clear main significant effects for both temperature (χ^2_2= 76.015; p < 0.001) and mating system (χ^2_1= 58.760; p < 0.001). Running models separately for each temperature level, mating system affected reproductive ageing at 20°C (χ^2_1= 12.107; p < 0.001; coefficient = 7.9 with a 95% CI of 3.49 to 12.49), at 24°C (χ^2_1= 35.324; p < 0.001; coefficient = 17.2 with a 95% CI of 11.53 to 22.88), and at 28°C (χ^2_1= 14.082; p < 0.001; coefficient = 11.8 with a 95% CI of 5.67 to 18.08).

Actuarial senescence- Survival analysis by means of a Cox proportional hazards model detected a significant temperature x mating system interaction for female survival (χ^2_2= 10.303; p= 0.005; Fig. 3), as well as main temperature (χ^2_2= 76.801; p < 0.001) and mating system effects (χ^2_1= 98.740; p < 0.001). Running models separately for each temperature level, mating system affected female survival at 20°C (χ^2_1= 43.426; p < 0.001; exponentiated coefficient = 1.87 with a 95% CI of 1.55 to 2.26), at 24°C (χ^2_1= 40.068; p < 0.001; exponentiated coefficient = 1.83 with a 95% CI of 1.52 to 2.22), and at 28°C (χ^2_1= 18.216; p < 0.001; exponentiated coefficient = 1.46 with a 95% CI of 1.23 to 1.74).

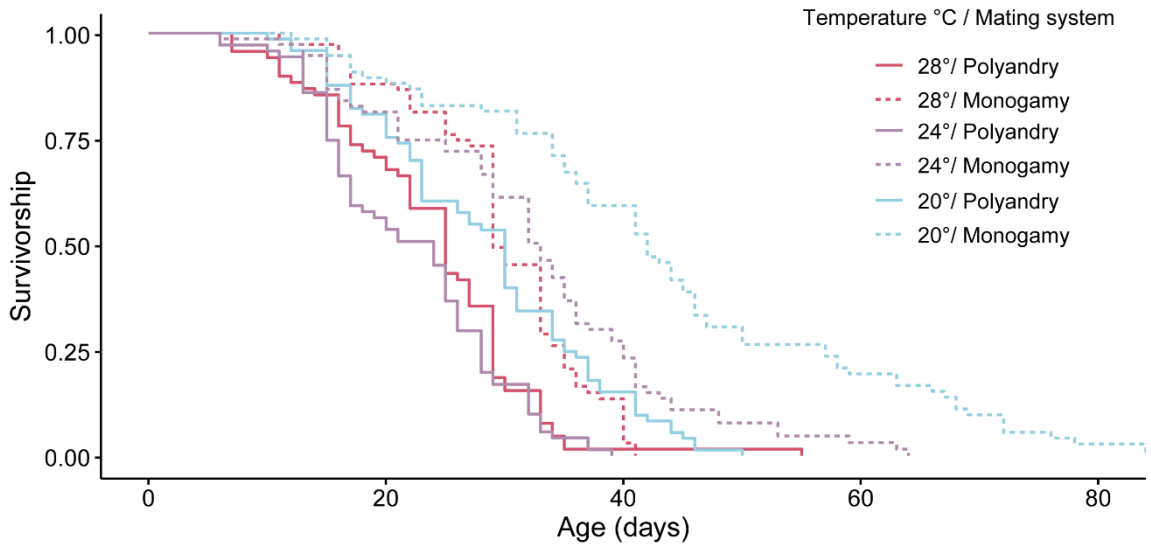

Figure 3. Male harm effect on female lifespan across temperature and mating system treatments.

<u>Reproductive behaviour</u>

The interaction between temperature and mating system was significant for aggression rate (χ^2_2= 15.043; p < 0.001), and we also detected main temperature (χ^2_2= 15.062; p < 0.001) and mating system effects (χ^2_1= 101.946; p < 0.001; Fig. 4a). Running models separately for each temperature level, mating system affected aggression rate at 20° (χ^2_1= 6.912; p= 0.008), at 24° (χ^2_1= 28.858; p < 0.001) and at 28° (χ^2_2= 32.311; p < 0.001). Likewise, we detected a significant temperature x mating system effect for courtship rate (i.e., number of courtships experienced by the female per hour) (χ^2_2= 15.915; p < 0.001), and clear main effects for both temperature (χ^2_2= 19.887; p < 0.001) and mating system (χ^2_1= 41.284; p < 0.001; Fig. 4b). Running models separately for each temperature level, mating system did not affect courtship rate at 20° (χ^2_1= 0.367; p= 0.545) but did at 24° (χ^2_1= 21.787; p < 0.001) and at 28° (χ^2_1= 40.136; p < 0.001). We also detected a significant interaction for female rejection rate (χ^2_2= 6.422; p= 0.040), as well as main temperature (χ^2_2= 18.791; p < 0.001) and mating system effects (χ^2_1= 25.561; p < 0.001; Fig. 4c). Running models separately for each temperature level, mating system did not affect female rejection rate at 20° (χ^2_1= 0.201; p= 0.653) but did at 24° (χ^2_1= 10.917; p < 0.001) and at 28° (χ^2_1= 19.27; p < 0.001).

<u>Ejaculate effects on female reproduction</u>

Female receptivity- The duration of the first mating in our female receptivity assays exhibited a clear sperm competition risk level x treatment duration interaction (χ^2_1= 5.947; p = 0.015), sperm competition risk level x temperature interaction (χ^2_2= 8.544; p = 0.014, Fig. 5a), and main effects for treatment duration (χ^2_1= 6.057; p = 0.014) and sperm competition risk level (χ^2_1= 36.738; p < 0.001). We found no significant main effect of temperature (χ^2_2= 4.260; p = 0.119). Running models separately for each temperature level, sperm competition risk level affected the duration of the first mating at 20°C (χ^2_1= 3.993; p = 0.045), at 24°C (χ^2_1=10.653; p = 0.001), and at 28°C (χ^2_1= 26.529; p < 0.001). Similarly, we detected a significant temperature x treatment duration interaction (χ^2_2= 6.593; p = 0.037), and a marginally non-significant sperm competition risk level x temperature interaction (χ^2_1= 5.423; p = 0.066; Fig. 5b) in female remating latency. We also detected significant main effects for treatment duration (χ^2_1= 4.026; p = 0.044), sperm competition risk level (χ^2_1= 4.494; p = 0.034) and temperature (χ^2_2= 9.408; p = 0.009). Running models separately for each temperature level, sperm competition risk level only affected remating latency at 28°C (χ^2_1=8.052; p = 0.004). At 20°C we found no significant sperm competition risk level effect (χ^2_1=0.950; p = 0.329), and the same for 24°C (χ^2_1=0.078; p = 0.779).

Female fecundity and survival- For the number of eggs produced by females during the three first days, we did not detect any significant interaction between factors: sperm competition risk level x treatment duration (χ^2_1= 0.039; p= 0.843), sperm competition risk level x temperature (χ^2_2= 1.911; p= 0.384), temperature x treatment duration (χ^2_2= 2.787; p= 0.248), neither main significant effect for temperature (χ^2_2= 2.742; p= 0.253) nor for sperm competition risk level (χ^2_1= 0.001; p= 0.968), but we did for treatment duration (χ^2_1= 9.009; p= 0.002). Likewise, for the total number of offspring produced by females during the days 1, 2, 3, 4, 5, and 8 after mating, we did not find any significant interaction between factors: male competition level x treatment duration (χ^2_1= 0.034; p = 0.852), male competition level x temperature (χ^2_2= 0.520; p = 0.770), temperature x treatment duration (χ^2_2= 0.972; p = 0.614). Total offspring did not differ significantly between temperatures (χ^2_2= 2.142; p = 0.342), nor between sperm competition risk levels (χ^2_1= 0.326; p = 0.567), but did differ between treatment durations (χ^2_1= 5.490; p = 0.019). Finally, survival analysis by means of a Cox proportional hazards model did not detect any significant interaction between factors: sperm competition risk level x treatment duration (χ^2_1= 0.012; p = 0.912), sperm competition risk level x temperature (χ^2_2= 0.463; p = 0.793), temperature x treatment duration (χ^2_2= 2.190; p = 0.334). However, we detected a significant main temperature (χ^2_2=10.189; p = 0.006) and treatment duration effects (χ^2_1= 114.030; p < 0.001). We did not detect a significant main sperm competition risk level effect (χ^2_1= 3.255; p = 0.071).

DISCUSSION

We show that male harm exhibits complex plasticity in response to temperature changes within an optimal reproductive range (20ºC-28ºC) for a wild *D. melanogaster* population, with several implications for our understanding of how sexual conflict unfolds in nature. First, we show that net harm to females varies markedly within this range, such that relative harm is maximal at 24ºC (H = 0.36), decreases at 20ºC (H = 0.22) and is not statistically detectable at 28ºC (H = 0.10). Second, rate-sensitive fitness estimates predict that population costs are dependent on population demography, so that male harm impacts population growth differently at cold vs. warm temperatures depending on whether populations are decreasing or growing. Third, our results strongly suggest that the male harm effect on population growth has to do with the fact that different mechanisms exhibit qualitatively different reaction norms in response to temperature, with distinct effects on different female fitness components. Thus, cold temperatures decreased courtship intensity (i.e., male harassment; pre-copulatory mechanism) and impacted female fitness mainly via accelerated actuarial

senescence. In contrast, warm temperatures modulated male ejaculate effects on female reproduction (i.e., receptivity), and impacted female fitness mainly via accelerated reproductive senescence. We discuss how such plasticity may affect how male harm impacts populations, as well as selection, adaptation and, ultimately, evolutionary rescue under a warming climate.

Temperature effects on male harm and its consequences for populations

We found that temperature variation within the optimal reproductive range for our study population in the wild had a strong effect on male harm levels. To gauge male harm, we used the standard procedure of comparing female LRS in monogamy, which imposes low male-male competition and limits sexual conflict, vs. polyandry (i.e., a female with three males), which imposes high male-male competition and intensifies sexual conflict between the sexes (Yun et al., 2021). Importantly, a recent study suggests that these sex ratios are common in mating patches in the wild, and are actually representative of the extremes in natural levels of male-male competition (Dukas, 2020). In monogamy, temperature did not affect female fitness (Fig. 1), showing that female reproduction is indeed optimal within this range. In contrast, the net decrease in female LRS in polyandry vs. monogamy was highly dependent on the thermal environment, with an average decrease of H = 0.36 at 24ºC, H = 0.22 at 20ºC and H = 0.10 at 28ºC, at which temperature we did not find a statistically significant effect of mating system on female LRS (Fig. 1).

Male harm effects are expected to be cumulative over time, so that their impact on female survival and reproductive output is likely not constant across female lifespan (Bonduriansky et al., 2008; Filice et al., 2020). At the same time, early vs. late life reproduction weigh differently on both individual fitness and how this impacts background population growth, depending on whether such population is decreasing, stable or growing (Edward et al., 2011; Priest et al., 2008). Thus, in order to evaluate how male harm is likely to impact populations across different temperatures, we calculated rate-sensitive fitness estimates for individual (Fig. 2a) and population (Fig. 2b) fitness across a range of demographic scenarios (i.e., decreasing, stable and growing populations; Edward et al., 2011). Overall, the impact of male harm was higher in decreasing populations, where late-life reproduction gains importance, which is consistent with the idea that male harm effects are cumulative. Above and beyond this general effect, the observed interaction between temperature and female fitness is maintained irrespective of population demography. That is, male harm decreases

female individual fitness more at 24ºC than at 20ºC and 28ºC. Interestingly, though, temperature also has a clear effect on how demography affects population-level costs. At hot temperatures the relative population costs of male harm vary considerably less with demography (i.e., background population growth) compared to colder temperatures (Fig. 2b). For a decreasing population, the relative population costs of male harm are significantly higher at 20ºC than at 28ºC, but this difference wanes as population growth rate (r) increases, to the point of reverting in a rapidly growing population (Fig. 2b). This suggests that male harm has more impact on late-life female fitness at cold temperatures, and hints at the possibility that cold vs. hot temperature affect qualitatively distinct parameters of female fitness, and underlying mechanisms of male harm.

Looking at effects on separate female fitness components yields results largely in agreement with the above ideas. Again, consistent with the fact that harm needs to accumulate in time to impact female fitness, temperature had no effect on how or whether male competition impacted early reproductive rate. Temperature did, however, modulate how male harm impacted on female actuarial vs. reproductive ageing. We found clear differences in female lifespan across temperature and mating systems treatments. Male harm effects on actuarial ageing (an increase in mortality rate with age) were more severe at 20°C (35% decrease in female lifespan) than at 24°C (31% decrease) and at 28°C (22% decrease; Fig. 3). In contrast, while male harm accelerated reproductive senescence at all temperatures, this decrease was more marked at 24°C (coefficient = 17.2 with a 95% CI of 11.53 to 22.88) and at 28°C (coefficient = 11.8 with a 95% CI of 5.67 to 18.08) than at 20°C (coefficient = 7.9 with a 95% CI of 3.49 to 12.49; Fig. SI1). In accordance with available evidence in lab-adapted flies (García-Roa et al., 2019), these results show that temperature does not seem to have a linear effect on ageing processes.

The above results open up the intriguing possibility that warm climates may lessen the impact of sexual conflict on population viability, perhaps facilitating evolutionary rescue. Male harm effects were found to be relatively lower in warmer temperatures and in decreasing populations, precisely the type of context that would be typical of a climate-change scenario. Similar results have been reported previously but in response to stressful temperatures. For instance, temperature has been used to induce environmental stress in natural populations of seed beetles (*Callosobruchus maculatus*), showing that a stressful thermal regime reduces intra-locus sexual conflict by aligning selection in males and females (Berger et al., 2014, but see Martinossi-Allibert et al., 2019). A previous study in a lab-

adapted population of *D. melanogaster* suggests male harm levels decrease when subject to maladaptive warm temperatures (García-Roa et al., 2019). A further consequence of the temperature sensitivity of male harm is that its net fitness consequences might be considerably lower than expected from past research when considered in its natural thermal setting. Our work thus joins an increasing number of recent studies in highlighting the importance of evaluating more ecologically realistic scenarios in sexual conflict research, particularly how natural fluctuations in the ecology of the socio-sexual context may affect sexual conflict processes (García-Roa et al., 2019, 2020; Gomez-Llano et al., 2018; Yun et al., 2017).

Temperature effects on sexual conflict mechanisms in Drosophila melanogaster

Prior to mating, *D. melanogaster* males harm females via sexual harassment, due to protracted courtship of one or several males that results in physical damage, interference with other behaviours (e.g., foraging or egg-laying) and costly energetic investment into male avoidance (e.g., female rejection) (Bretman & Fricke, 2019; Partridge & Fowler, 1990; Teseo et al., 2016). Importantly, previous studies in this species have shown that male harm is directly related to courtship intensity and female rejection, and indirectly to male-male aggression as a direct measure of male intrasexual competition (e.g., Bretman & Fricke, 2019; Carazo et al., 2014; Partridge & Fowler, 1990). In our study, we found a clear increase in both courtship intensity and female rejection in polyandry vs. monogamy, but this was largely dependent on the thermal environment (Fig. 4b-c). While we found clear evidence that harassment increases at polygamy at 28°C, and 24°C, we did not find a similar effect at 20°C (Fig. 4b). Female rejection behaviours exhibited the exact same trend (Fig. 4c). Thus, our results suggest that male harassment decreases drastically at cold temperatures and is perhaps maximal at warm temperatures, at which temperatures we also detected the highest level of male-male aggression (Fig. 4a). The above results seem to suggest that the decrease in male harm to females that we detected at 20ºC vs. 24ºC (Fig. 1) is likely explained by a drastic drop in male harassment of females at this temperature.

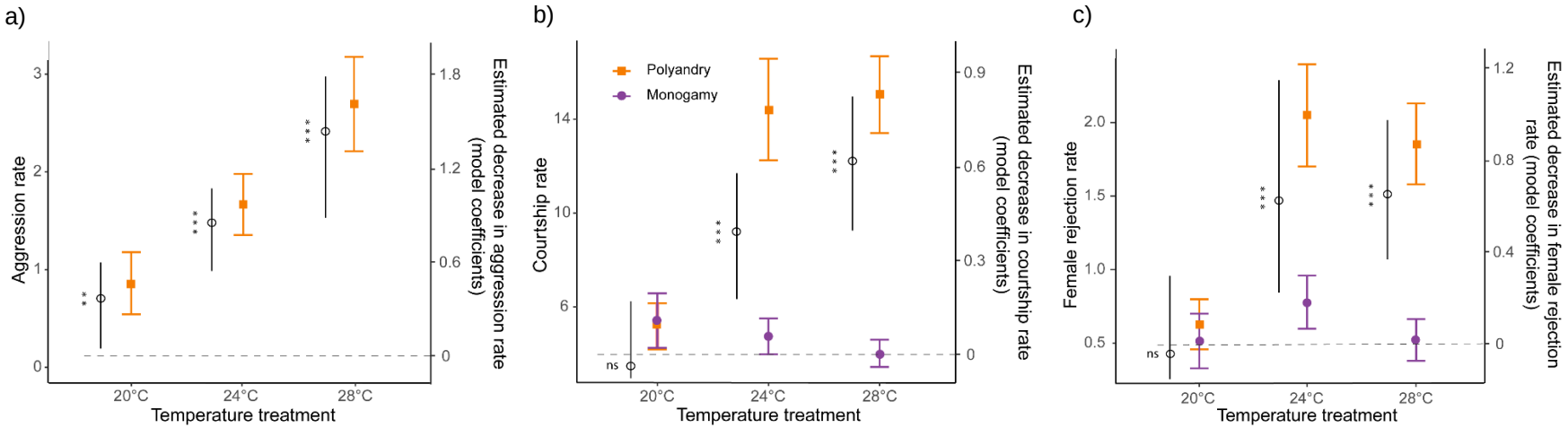

Figure 4. Reproductive behaviours (mean ± SEM) across temperature and mating system treatments. a) aggressions male-male per hour, b) courtships per female per hour, and c) female rejections per hour. Second axis represents the model coefficient estimates (95% CI) for the estimated variation in aggression, courtship and female rejection rate that ensue with polyandry vs. monogamy, across the different temperature treatments. Estimates are coefficients from GLMs fitted separately for the three temperature treatments.

During mating, *D. melanogaster* males transfer seminal fluid proteins that manipulate female re-mating and egg-laying rates to their advantage, but at a costs to females in terms of lifespan and lifetime reproductive success (Chapman, Bangham, et al., 2003b; Chapman et al., 1995; Sirot et al., 2009; Wigby & Chapman, 2005). Furthermore, these effects are known to be modulated by the socio-sexual context, such that males strategically adjust their investment in SFPs depending on expected sperm competition risk (SCR) levels (Bretman, Fricke, & Chapman, 2009; Hopkins et al., 2019; Wigby et al., 2009). We run a series of assays to investigate whether temperature modulates any of the known phenotypic effects that SFPs have on females following a single mating (note these assays are standard for *D. melanogaster*; e.g., Chapman et al., 1995; Perry, Sirot, & Wigby, 2013; Wigby & Chapman, 2005; Wigby et al., 2009) with males that were exposed to low (i.e., kept alone in their vial) or high sperm competition risk (i.e., kept with 7 other males in a vial). The temperature males were kept at prior to mating did not modulate how mating affected short-term female fecundity or lifespan, but we did detect a clear effect of temperature with respect female receptivity. Generally, reception of a male ejaculate resulted in a sharper decrease in female receptivity (i.e., longer remating latency) when males perceived a high SCR, which is to be expected and is in accordance with previous studies (Bretman et al., 2009; Bretman, Fricke, Hetherington, Stone, & Chapman, 2010; Denis et al., 2017). However, this effect was largely modulated by temperature, such that it was only clearly detected at 28ºC (Fig. 5b). Interestingly, this effect was consistent for males treated for 48h and 13 days and was paralleled by mating duration (Fig. 5a). Namely, males exposed to high SCR generally mated for longer than males exposed to low SCR, again in line with previous findings (Bretman et al., 2010; Bretman, Westmancoat, Gage, & Chapman, 2013), but this difference was clearly larger at 28ºC (Fig. 5a). It is worth restating that, under monogamous conditions, we did not detect a decrease in female productivity at 28ºC vs 24ºC and 20ºC, so that general differences in sperm viability are unlikely to account for the aforementioned differences. All in all, our results suggest that at least some post-copulatory harm mechanisms are sensitive to temperature, and that receipt of a male ejaculate results in a sharper decrease of female receptivity at warm temperatures. We speculate that this may contribute to explain why male harm drops so sharply at 28ºC despite the fact that male harassment and male-male competition seems to be maximal at this temperature. In *D. melanogaster*, as in many other species, repeatedly mating is costly for females in terms of lifetime reproductive success (Arnqvist & Nilsson, 2000; Chapman et al., 1995; Fowler &

Partridge, 1989). An intriguing possibility is thus that SFPs are more effective at lowering female re-mating rates at warm temperatures, thereby buffering these costs. We suggest future studies should look into this by examining in detail how temperature affects the composition and transfer of SFPs to females.

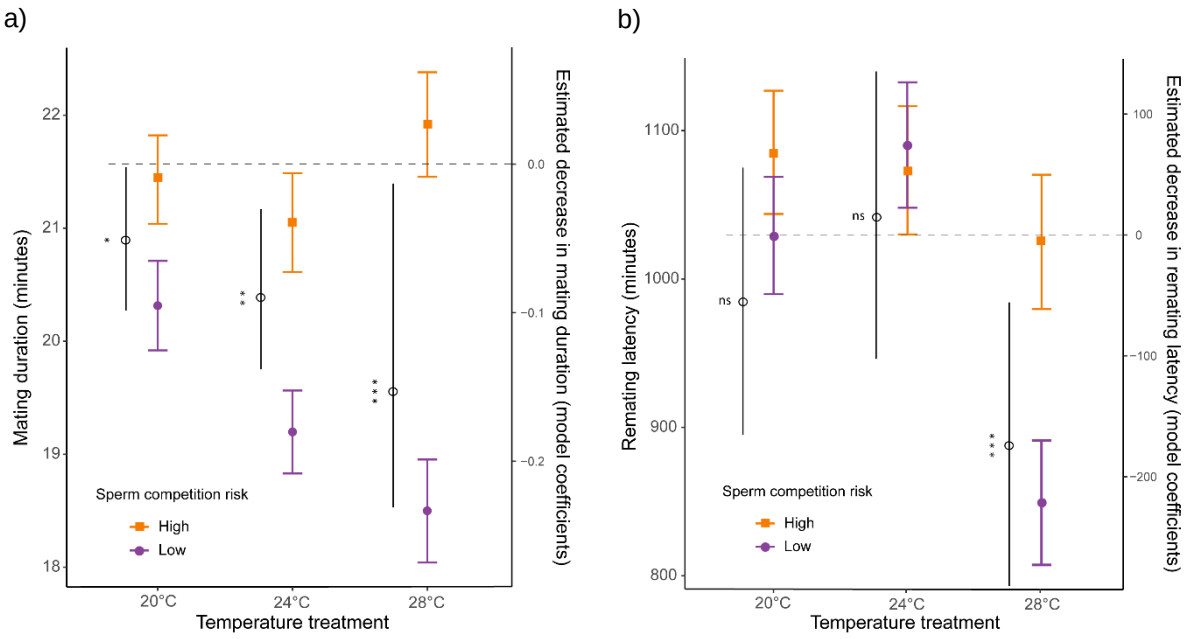

Figure 5. a) Mating duration of males exposed to a high (8 males per vial) or low sperm competition risk (1 male per vial) level 48 hours and 13 days before mating across temperature treatments, b) females remating latency following a single mating with either a male from a high or low sperm competition risk level 48 hours and 13 days before mating across temperature treatments. Second axis represents the model coefficient estimates (95% CI) for the estimated variation in mating duration and remating latency that ensue with high vs. low sperm competition risk, across the different temperature treatments. Estimates are coefficients from GLMs fitted separately for the three temperature treatments.

Conclusions

Our findings may have implications for our understanding of how sexual conflict unfolds in nature, and its consequences for populations. First, they add to growing evidence (Gomez-Llano et al., 2018; MacPherson et al., 2018; Malek & Long, 2019; Perry & Rowe, 2018; Yun et al., 2017) indicating that ecological context is key in shaping sexually antagonistic coevolution and, in particular, suggest that temperature may be a particularly salient ecological factor to understand how sexual conflict evolves and operates in nature (García-Roa et al., 2020). Second, they highlight that male harm mechanisms can be highly plastic even in response to relatively minor fluctuations in temperature well within the optimal

reproductive range, and suggest that different harm mechanisms are differently affected by temperature. Third, they suggest that male harm effects on female life-history and fitness components are asymmetrically modulated by temperature; male harm particularly decreased survival at cold and moderate temperatures, and reproductive ageing at moderate and hot temperatures. In conjunction, these phenomena may have a bearing on evolutionary rescue and local adaptation processes. For example, in maintaining genetic variation in sexually selected traits in males, and/or in ameliorating the demographic impact of sexual conflict in populations facing environmental change. In this sense, our results hint at the intriguing possibility that rapid warming, such as human-induced climate change, may have unexpected positive consequences via its effect on male harm, potentially facilitating evolutionary rescue. We thus suggest two priorities for future research on this area are: a) studying the effect of temperature on post-copulatory mechanisms (e.g., seminal fluid accessory glands proteins and their effects in *Drosophila*), and b) developing formal models to explore under what contexts might temperature effects on male harm facilitate evolutionary rescue.

MATERIALS AND METHODS

Field Collection

In October 2018, we used banana traps to sample *Drosophila melanogaster* flies from three wineries in Requena (Spain): “Hispano-Suizas” (39.466128, -1.149642), “Pago de Tharsys” (39.497834, -1.122781) and “Dominio de la Vega” (39.515079, -1.143757). Traps were setup within the wineries, but in premises that were open to the exterior and to ample surrounding vineyards and/or in the vineyards themselves. After collection, we anaesthetized flies using mild CO_2 exposure. We then separated and individually distributed field-collected females in vials with standard food, left them to lay eggs for a period of 48 hours and then incubated their eggs at 24ºC, 60% humidity and a 12:12 dark-light cycle for 14 days to allow adult flies to emerge. We inspected the genital arch of F1 males of each of these female isolines to distinguish *D. melanogaster* isolines from *D. simulans*. We then collected three males and three females from each *D. melanogaster* isoline (total of 276 flies from 46 isolines) and released them into a population cage with a surplus of food medium supplemented with live yeast, setting up the “Vegalibre (VG)” population. In November 2019 and October 2020, to maintain natural variation, we re-sampled the wineries and added 348 and 756 flies from 58 and 126 isolines, respectively, following the same procedure (27

isolines from “Pago de Tharsys” and 31 isolines from “Dominio de la Vega” in November 2019 and 33 isolines from “Pago de Tharsys”, 50 isolines from “Dominio de la Vega” and 43 isolines from “Hipano-Suizas” in October 2020).

Stock maintenance and acclimation

We carried out all experiments between March 2020 and April 2021, using individuals from the VG field population kept in the laboratory with overlapping generations at an average temperature of 24°C with daily fluctuations (±4°C) that mimic natural daily temperature conditions during the reproductively active season, at ~60% humidity and on a 12:12-hr light: dark cycle. We used maize-malt medium (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) throughout. We used the following procedure to collect experimental flies. We introduced yeasted grape juice agar plates into stock populations to induce female oviposition. We then collected eggs and placed them in bottles containing ~75ml of medium to be incubated at 24 ± 4°C at a controlled density (Clancy & Kennington, 2001). We collected virgin flies within 6h of eclosion, under ice anaesthesia, and then we sexed and kept them in vials with food until their use (~3 days), at 24 ± 4°C.

Fitness assays: quantifying male harm

To study whether male harm is affected by temperature, we established a factorial design to measure survival and lifetime reproduction success (LRS) of female flies under monogamy (i.e., one male and one female per vial) vs. polyandry (i.e., three males and one female per vial), across three stable temperature treatments typical of this population during their reproductively active period in the wild: 20, 24, and 28°C. Comparison of female fitness at monogamy vs. polyandry is a common way to gauge male harm in *Drosophila* and other organisms (Yun, Agrawal, & Rundle, 2021), and our treatments reflect the low and high-end of the spectrum of sex ratios that are typical of *D. melanogaster* at mating patches in the wild (Dukas, 2020).

We first randomly divided virgin flies into three groups that we allocated to the three different stable temperature treatments 48 hours before starting the experiment. Flies remained at those temperatures until the end of the experiment. To estimate LRS, we transferred flies to fresh vials twice a week using mild CO_2 exposure anaesthesia. We incubated the vials

containing female eggs at 24 ± 4°C for 15-20 days (~15 days for 28°C, ~17 days for 24°C and ~20 days 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 developmental time because of temperature differences in the first 3-4 days of each vial (i.e., before flipping females to new fresh vials). We discarded and replaced males by young (2 to 4 days old) virgin males three weeks after starting (at the same time for all treatments). We kept a stock of replacement males maintained at each of the three temperatures (20, 24 and 28°C) to replace dead male flies if needed. We kept flies under these conditions during six weeks, after which we discarded males and followed females until they died. We started the experiment with 468 females (78 per each temperature x mating system treatment) and 936 males (234 per each temperature x polyandry and 78 per each temperature x monogamy). Final sample sizes were: a) at 20°C: 74 females for polyandry and 76 females for monogamy; b) at 24°C: 72 females for polyandry and 77 females for monogamy; c) at 28°C: 70 females for polyandry and 75 females for monogamy. Differences between start and final sample sizes across treatments are due to discarded/escaped flies since the start of the experiment. We estimated the overall degree of male harm by calculating the relative harm (H) following Yun et al. (2021):

$$H = \frac{W_{monogamy} - W_{polygamy}}{W_{monogamy}}$$

Finally, and using the data collected above, we partitioned overall effects on LRS into effects of temperature x mating system (i.e., male-male competition level) on early reproductive rate (i.e., offspring produced during the first two weeks of age) and reproductive (i.e., offspring produced over weeks 1-2 vs. 3-4) and actuarial senescence (i.e., survival).

Behavioural assays: quantifying male-male aggression and harassment of females

Immediately after the fitness experiment started, we conducted behavioural observations on the first day of the experiment across all treatments, to investigate the behavioural mechanisms that might underlie the potential fitness effects evaluated above. Due to logistic limitations, we conducted behavioural observations in the same temperature control room, so we had to conduct trials at 20, 24 and 28°C in three consecutive days (with both monogamy and polyandry treatments evaluated at the same time for each temperature), in randomized order (i.e., 20, 28 and 24°C). However, all flies were 5 days-old at the start of the experiment. We measured the following behaviours: (a) courtship intensity (number of

courtships experienced by a female per hour), (b) male-male aggression behaviours and (c) female rejection behaviours (Bastock & Manning, 1955; Connolly & Cook, 1973). We also recorded the number of total matings during the observation period.

Observations started at lights-on (10 a.m.) and lasted for 8 hr, during which time we continuously recorded reproductive behaviours using scan sampling of vials. Each complete scan lasted approximately 8 min, so that we always conducted one complete scan every 10 min to ensure recording all matings (see below). Scans consisted in observing all vials in succession for ca. 3s each and recording all occurrences of the behaviours listed above. We interspersed these behavioural scans with very quick (<1 min) mating scans where we rapidly swept all vials for copulas at the beginning, in the middle and at the end of each behavioural scan. This strategy ensured that we recorded all successful matings (>10min), which typically last between 15 and 25min in our population of *D. melanogaster*, during our 8-hr observation. We obtained a total of 49 scans per vial. Behavioural observations were conducted only once, on day 1 of the fitness experiment, as prior experiments have shown that courtship, aggressive and female rejection behaviours are stable over time in *D. melanogaster*, so that our behavioural indexes are representative of long-term treatment differences (e.g., Carazo, Perry, Johnson, Pizzari, & Wigby, 2015; Carazo, Tan, Allen, Wigby, & Pizzari, 2014). In contrast to courtship and aggression indexes, note that total mating frequency over the first day cannot be taken as a reliable measure of mating rate (Wolfner, 1997), and thus our rationale in recording this variable was just to ensure that early mating ensued normally across treatments (which was the case, see SI2).

Female reproduction and survival assays: quantifying male ejaculate "toxicity"

To examine post-mating mechanisms that might underlie the fitness effects observed in our first experiment, we conducted four additional assays to test whether temperature modulates the previously well-documented effects of male ejaculates on female reproduction and survival in *D. melanogaster*. Briefly, males of this species transfer seminal fluid proteins (SFPs) produced by their accessory glands that increase short-term female fecundity, as well as decrease female receptivity and survival (Chapman, Liddle, Kalb, Wolfner, & Partridge, 1995; Wigby & Chapman, 2005). In addition, prior studies have shown that males are able to tailor investment into SFPs according to the expected sperm competition risk and intensity (Hopkins et al., 2019). Thus, we set-up a factorial design where we manipulated the temperature (i.e., 20, 24 and 28ºC) and perceived sperm competition risk levels (i.e.,

males kept alone vs. with 7 more males in a vial) at which adult males were kept prior to mating, for two temperature treatment durations (i.e., 48 hours or 13 days), and then measured how reception of their ejaculate in a common garden environment (i.e., 24ºC) affected female fecundity, survival and reproduction.

Receptivity assays- We first collected experimental males as virgins (i.e., within 6 h of eclosion) under ice anesthesia and randomly placed them either individually or in a same-sex group of 8 in medium containing plastic vials. Next, we randomly divided them into three groups that we allocated to the different stable temperature treatments for either 48 hours (experiment 1) or 13 days (experiment 2) immediately before the beginning of each experiment. In experiment 2 (treatment duration of 13 days), we emptied the seminal fluid of experimental males before we allocated them to the different temperature / competition treatments. To do so, we housed individually experimental males with four standard virgin females for 24 hours. This strategy ensured that spermatogenesis took place over the 13 days under the temperature / competition treatments.

We collected all females and competitor males used in receptivity assays as virgins and held them in groups of 15 to 20 flies at 24 ± 4°C. Experiments started by exposing all virgin females to single experimental males for 2.5 h at 24°C. After a successful copulation, we separated the mated females from the males and kept them individually in medium containing vials until the next mating trial. We discarded unmated females and experimental males. 72 h after the first mating, we individually exposed females to single virgin competitor males for 12 h. After each trial, we transferred unmated females into a new medium containing vial, until the next mating assay 24 h later. We repeated remating trials every 24 h for three consecutive days. We calculated the cumulative percentage of remated females for each of the three days of each experiment. We conducted the experiments in two blocks each: with n = 390 females for each batch in experiment 1 (n = 436 rematings) and n = 420 females for each batch in experiment 2 (n = 676 rematings). We also recorded mating duration for the first mating and mating latency (i.e., time between males being introduced into the female-containing vial and copulation) and mating duration for re-matings. Females that did not remate within those three days were right-censored. Females and all experimental males were 4 days old at the start of experiment 1. In experiment 2, females and “re-mating” competitor males were 4 days old, while experimental males were 18 days old.

Fecundity and survival assays- To gauge effects on female short-term fecundity and long-term survival, we performed two experiments (experiments 3 and 4) where we compared the oviposition and egg fertility of females mated with male flies subject to the same factorial design imposed in receptivity experiments. We collected and treated all experimental males as in the receptivity assays described above, and then proceeded to mate ~4 day-old virgin females in single pairs to either 4- (experiment 3, 48h temperature treatment duration) or 18-day old experimental males (experiment 4, 13d temperature treatment duration) for 2.5h at 24°C. After copulation, we separated mated females from males and kept them individually in medium containing vials. We discarded unmated females. We then transferred females to fresh vials every 24h for 4 days, and then every 3 days twice. Later, we transferred female flies to fresh vials once a week, and we combined vials to maintain density at ten flies per vial. We kept female flies under these conditions until they died. We removed dead flies at each transfer and recorded female deaths. We counted eggs laid the first 3 days and vials from days 1, 2, 3, 4, 5, and 8 after mating were retained to count progeny to determine egg viability. We tested 545 females at the starting point of the experiment 3, and 480 females at the starting point of the experiment 4.

Statistical analyses

We performed all statistical analyses using R statistical software (version 3.5.2). In all models, we assessed fit by visual inspections of diagnostic plots on raw and residual data (Zuur, Ieno, & Elphick, 2010). To examine temperature effects on male harm, we evaluated the interaction between mating system and temperature on female fitness (LRS), survival and male and female reproductive behaviours. We fitted generalized linear models (GLMs) with temperature, mating system and their interaction as fixed effects. We also used Cox proportional hazards survival model to analyse potential differences in mortality risk across treatments, using the *survival* and *survminer* packages, including the females lost during manipulations as “right censored” individuals (i.e., individuals that are taken into account for demographic analysis until the day they disappear, Kleinbaum & Klein, 2012). We also censored any remaining females after the six weeks of the experiment. Graphical inspection of LRS and reproductive behaviours (courtship intensity, intersexual aggression behaviours and female rejection) revealed that the normality assumption was apparently violated, as well as the independence assumption for LRS. Box–Cox transformation (Quinn & Keough, 2002) solved these problems and allowed us to run a GLM with a Gaussian error distribution. In all these afore mentioned cases, fitted models were subsequently validated. In addition,

we compared GLMs with their corresponding null GLMs using likelihood ratio test. In all models, we used ANOVA type III to compute *p*-values. We detected a problem of collinearity between mating system and the interaction in the LRS model. We fitted the model again without the main mating system effect (which was not our main interest) and also ran models separately for each temperature level. As a complementary analysis, we ran a model with temperature as factor with a quadratic contrast table predetermined (note that the relation between LRS and temperature does not seem to be linear, Fig 1), obtaining similar results. For the total matings recorded across the 8h observation period (i.e., mating rate), we initially used a GLM with Poisson errors. However, the dataset contained many zero values. We therefore analysed the data using a Hurdle model, in which the zero values are modelled separately from the nonzero values (Zuur et al., 2010). Additionally, we compared GLM and Hurdle models using an information-theoretic approach based on Akaike's Information Criterion (AIC). The minimum AICc value indicates the best supported model given the trade-off between fit to the data and model complexity (Konishi & Kitagawa, 2008). To estimate how male harm impact estimated population growth across varying demographic scenarios (i.e., decreasing, stable and growing populations), based on our LRS measures, we calculated rate-sensitive fitness estimates (Edward, Fricke, Gerrard, & Chapman, 2011) against different population background growth rates of $r = -0.1$, $r = -0.05$, $r = 0$, $r = 0.05$ and $r = 0.1$.

For receptivity (mating duration and remating latency) and fecundity (total adults) analyses, we fitted generalized linear models (GLMs) with temperature, sperm competition risk level, treatment duration and their interaction as fixed effects. For egg production data, we fitted a generalized linear mixed model (GLMM) with temperature, sperm competition risk level, treatment duration and their interaction as fixed effects and day number as a random effect, with a zero inflated distribution, in which the zero values are modelled separately from the nonzero values (Zuur et al., 2010). We assessed significance of factors by dropping individual terms from the full model using the "drop1" function, refitting where the interaction was nonsignificant. We also used Cox proportional hazards survival model to analyse potential differences in mortality risk across treatments, using the *survival* and *survminer* packages, including the females lost during manipulations as "right censored" individuals. Finally, we also ran models separately for each temperature level for the cases in which a significant interaction between sperm competition risk level and temperature treatment was detected.

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COMPETING INTERESTS

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