

Experimental evolution reveals that males evolving within warmer thermal regimes improve reproductive performance under heatwave conditions in a model insect

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Miscellaneous Information

Ethics

This study was approved by, and followed strict guidelines to, the University of East Anglia's Animal Welfare and Ethical Review Board.

Data accessibility

Datasets are available from GitHub Digital Repository (<https://github.com/rvasudeva83/ThermalAdaptation.git>).

Authors' contributions

M.J.G. conceived the project, M.J.G., K.S and R.V. designed the experiments. K.S. and R.V. maintained the experimental evolution lines and conducted empirical work. Data were analysed by K.S. K.S. wrote the manuscript with additional input and editing R.V. and M.J.G.

Competing interests

The authors declare no competing or financial interests.

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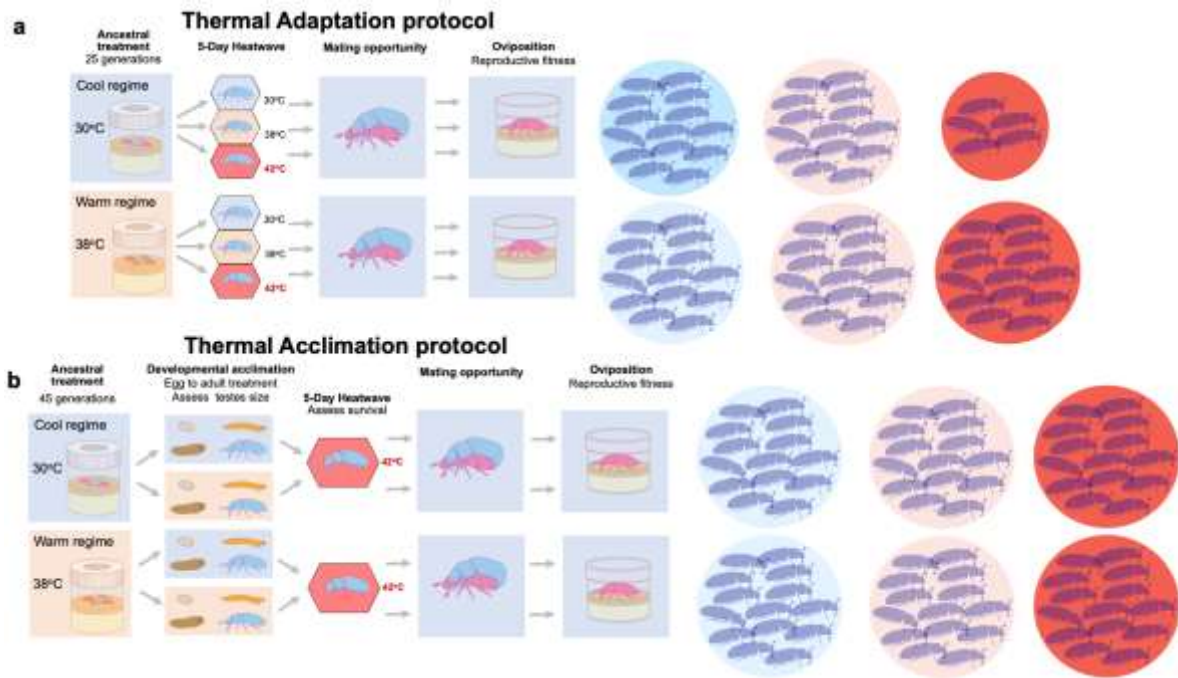
Abstract

Climate change is increasing mean temperatures, and intensifying heatwaves. Natural populations may respond to stress through shorter-term acclimation via plasticity and/or longer-term inter-generational evolution. However, if the pace and/or extent of thermal change is too great, local extinctions occur; one potential cause in ectotherms is identified to be the heat-liability of male reproductive biology. Recent data from several species, including the beetle *Tribolium castaneum*, confirmed that male reproductive biology is vulnerable to heatwaves, which may constrain populations. However, such reproductive-damage may be overestimated, if there is potential to adapt to elevated mean temperatures associated with climate change via evolution and/or acclimation. Here, we tested this to evaluate whether pre-exposures could improve heatwave tolerance (adaptation or acclimation), by experimentally evolving *T. castaneum* populations to divergent thermal regimes (30°C versus 38°C). Findings across assays revealed that relative to 30°C-regime males, males from the 38°C regime, maintained constantly at 8°C warmer for 25 generations, displayed an increase; i) in post heatwave (42°C) reproductive fitness by 55%, ii) survival by 33% and iii) 32% larger testes volumes. Unexpectedly, in the acclimation assay, warm-adapted males' post-heatwave survival and reproduction were best if they experienced cool developmental acclimation beforehand, suggesting a cost to adapting to 38°C. These results help progress knowledge of the potential for survival and reproduction to adapt to climate change; trait specific adaptation to divergent thermal regimes can occur over relatively few generations, but this capacity depended on the interaction of evolutionary and thermal acclimatory processes.

Keywords: Adaptation, Acclimation, Climate change, Testes, Experimental, Fertility, Heatwaves, Insect, Reproduction

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Graphical Abstract



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1. Introduction

The Earth's climate is warming, and by 2100 mean global temperatures will likely rise beyond 2°C relative to pre-industrial conditions (IPCC, 2021; Raftery et al., 2017). As mean temperature increases, so too does its variability (Sexton and Harris, 2015; Wiel and Bintanja, 2021), with extreme temperature events like heatwaves expected to become longer, more variable, hotter and more frequent (Christidis et al., 2015; Hobday et al., 2016; IPCC, 2021; Meehl and Tebaldi, 2004; Perkins et al., 2012). The definition, regional metrics of heatwave varies (e.g., Perkins-Kirkpatrick and Lewis, 2020), but often incorporates a period of abnormally hot weather relative to a reference threshold, which can last from 2 days to months (IPCC, 2021). Here, we focus on Frich et al.'s (2002) definition of daily temperatures exceeding the long-term average by at least 5°C for at least 5 days.

Species respond to climate change partly because temperature is a fundamental abiotic variable (Bellard et al., 2012) which influences cells' structural integrity, metabolism and water balance (Frazier et al., 2006; Willmer, 1982). Commonly, life-history traits express a negatively-skewed thermal performance curve; with a gradually accelerating increase in performance from a critical thermal minimum (CT_{min}) to a critical thermal optimum (CT_{opt}), the temperature where a trait performs at its best, then rapid decline to a critical thermal maximum (CT_{max}), beyond which the trait becomes dysfunctional (Angilletta et al., 2010; Terblanche et al., 2007). General patterns across metazoans seems that the ancestral CT_{opt} clusters around 20°C, CT_{min} varies between 17°C and -70°C, whereas the CT_{max} only between 15°C and 47°C (Hoffmann et al., 2003). However, lifecycle completion often requires a narrower range such as 10°C to 30°C for insects (Angilletta, 2009; Gibbs and Eran, 2009). Natural populations' stress responses, from temperatures encroaching their CT_{max} s, have been extensively recorded over decades (Root et al., 2003; Ummenhofer and Meehl, 2017; van de Pol et al., 2017); examples include range shifts (Chen et al., 2011), phenological changes (Cohen et al., 2018), metabolic adjustments (Bickford et al., 2010) and physiological alterations (Sheridan and Bickford, 2011; Zeuss et al., 2014).

Climate-induced extinctions are a phenomena which are widely reported (Fox et al., 2014; Hallmann et al., 2017; Harvey et al., 2023; Lister and Garcia, 2018; Parmesan and Yohe, 2003). However, there has been '*disturbingly*' little knowledge and uncertainty around the biological mechanisms underlying such losses (Cahill et al., 2014, 2013; van de Pol et al., 2017). For example, Urban (2015) reviewed 131 studies, and calculated that on average 7.9% of species are threatened, however, predictions varied between 0 and 54%.

Common research frameworks on extinction often incorporate: 'exposure', 'vulnerability' and 'adaptive capacity' (Chen et al., 2011; Gienapp et al., 2008; Palmer et al., 2015; Willis et al., 2015). In the context of global warming, exposure equates to the rate of temperature increase and intensity of extreme values, vulnerability is a population's current conservation status, while adaptive capacity is the potential for a population to reduce its future vulnerability. Exposure and vulnerability have been relatively well explored using climate modelling approaches (Oliver et al., 2015; Palmer et al., 2015). However, there is uncertainty for long-term predictions and conservation efforts, because the adaptive capacities of various biological traits remains relatively unknown (Angilletta, 2009; Gienapp et al., 2008; Palmer et al., 2015; Sgrò et al., 2016). For example, extinction

rates may be underestimated if just CT_{max} and CT_{opt} metrics are assumed, as this might not be homogenous across populations/individuals. It might be overestimated if populations are assumed to have no adaptive capacity (Deutsch et al., 2008; Harte et al., 2004). Consequently, laboratory experiments have been recommended to address such knowledge gaps on the drivers of insect extinctions (Cahill et al., 2013; Hoffmann et al., 2003; Hufbauer et al., 2015; Maino et al., 2016).

Two broad mechanisms exist for phenotypes adapt to climate change. The first being evolution by natural selection applying a filter on existing allelic diversity, so that beneficial genes are selected for, and increase in frequency over generations (Bellard et al., 2012; Bijlsma and Loeschcke, 1997). The second adaptive mechanism operates through plasticity, where the phenotype expressed by an individual changes depending on the environmental stimuli previously encountered, without altering deoxyribonucleic acid (DNA)'s primary structure (Loeschcke and Sorensen, 2005; Sgrò et al., 2016). Beneficial plasticity can be divided into short acute hardening or more gradual acclimation; moreover, acclimation can be divided into conditioning individuals during their development, conditioning as adults, or preparing offspring intergenerationally through parental conditioning (Hoffmann et al., 2003; Terblanche et al., 2011). Generally, some researchers view plasticity as a fast time-buying mechanism until more persistent evolutionary selection can occur (Guillaume et al., 2016; Martinez et al., 2016). However, others researcher caution that plasticity can be a stress response with neutral (Woods and Harrison, 2002), or even a maladaptive effects (Franke et al., 2014).

The adaptive potential for increasing the CT_{max} of survival has been explored using a range of biology and diverse methodologies (Hoffmann et al., 2003; Terblanche et al., 2011). Often findings seem mixed, but show that increasing CT_{max} is relatively difficult (Bauerfeind and Fischer, 2014; Hoffmann et al., 2013). However, study of CT_{rng} for fertility seems overlooked (Dolgin et al., 2006; Fig. 2e Seebacher et al., 2015), despite male reproduction being established as vulnerable trait to temperature, directly linked to fitness, and a constraint on population viability (David et al., 2005; Porcelli et al., 2017; Walsh et al., 2019). The thermal liability of male fertility was recognised in endotherms decades before ectotherms (Setchell, 2006), despite the latter's ecological importance (Dicks et al., 2016), abundance (Stork et al., 2015) and dependence on external temperatures (Paaijmans et al., 2013).

Initially, extensive research in vinegar flies (*Drosophilidae*) revealed that the critical thermal range (CT_{rng}) of male fertility defined population viability, by being least 1-2°C narrower than for survival and female fertility (David et al., 2005; Rohmer et al., 2004). Subsequent research has highlighted that heat-sensitivity of male fertility seems widespread across ectothermic taxa (Dougherty et al., 2024; van Heerwaarden and Sgrò, 2021; Ørsted et al., 2024; Parratt et al., 2021; Rodrigues et al., 2022; Walsh et al., 2019; Weaving et al., 2024; Zwoinska et al., 2020), with examples across: Hymenoptera (Dávila and Aron, 2017; Stürup et al., 2013), Lepidoptera (Zhang et al., 2013; Liang et al., 2014), Diptera (Araripe et al., 2004; David et al., 2005), Coleoptera (Saxena et al., 1992; Vasudeva et al., 2014), Nematoda (Harvey and Viney, 2007; Prasad et al., 2011) and Annelida (Guillaume et al., 2016).

Here, the adaptive potential of male biology to increasing mean temperatures associated with climate change, and whether it could mitigate against detrimental impacts of heatwave extremes was assessed. This knowledge gap was tested using the powerful combination of experimental

evolution (reviewed in Garland and Rose, 2009; Hoffmann and Sgrò, 2011) and the red flour beetle (*Tribolium castaneum*) model insect. *T. castaneum* has several advantages as an amenable research model to investigate thermal adaptation (Mahroof et al., 2005a; Sokoloff, 1974). First, inter-species local thermal adaptation is apparent in natural populations of *Tribolium*, for example, *T. confusum*'s distribution is poleward of *T. castaneum*'s, and the former's CT_{opt} is lower for survival and developmental rate (Park and Frank 1948; Raros and Chiang, 1970; Fig. 10.1 Sokoloff 1974). Second, *T. castaneum* has intra-specific genetic variation in traits linked to temperature like $CT_{max/min}$ (Fig. 11.2 Sokoloff 1974), heat-sensitive mutants (Morgan, 1965), and morphology (Noh et al., 2016). Third, *T. castaneum* shows intra-population plasticity with temperature for behaviour (Amos et al., 1968; Park and Frank, 1948) and heat shock protein (HSP) expression (Mahroof et al., 2005c, 2005b; Schinko et al., 2012). Previously, ecologically relevant 5-day heatwave simulations (Frich et al., 2002; Mherrerera, 2016), 5-7°C higher than *T. castaneum*'s general CT_{opt} (Howe, 1956; Sokoloff, 1974), halved male reproductive fitness and damaged several aspects of fertility such as offspring production, sperm count, sperm storage and sperm viability (Sales et al., 2018, 2024).

Here, by experimentally evolving replicate populations from a common stock in divergent thermal regimes 8°C apart (30°C and 38°C regimes) through non-overlapping generations, we tested the prediction that 1) males from warmer populations (38°C regime) would have a relatively higher CT_{opt} and better performance near their CT_{max} than males from cooler populations (30°C regime and progenitor populations). Particularly, that at warm-regime males would have 1a) relatively larger testes at 38°C and that their 1b) post-42°C heatwave survival and 1c) post-heatwave reproductive fitness would be comparatively greater than cool-regime males. Conversely, thermal adaptation would not be apparent if 30°C and 38°C thermal regime males' traits performed similarly. Additionally, we tested 2) whether any apparent adaptation warm-regime males' 2a) survival, 2b) reproductive fitness or 2c) testes size to higher temperatures was specifically due to long-term inter-generational genetic evolution, or via short-term intra-generational acclimatory modifications during divergent developmental temperatures, using transplant experiments (following Dolgin et al., 2006; Hoffmann et al., 2013). Evolution would prevail if the higher inter-generational regime temperature treatment was more effective for the success of traits at higher temperatures, acclimation would be more influential if the immediate generation's exposure to higher temperature during development was more effective for trait success at high temperatures.

2. Materials and methods

2.1 General methods

General maintenance, *T. castaneum* biology, fitness assays, dissections, equipment, and heatwave simulation rationale have previously been described in full detail (Sales, 2019; Sales et al., 2018, 2021, 2024; Vasudeva et al., 2019, 2021). Maintenance of all populations involved in this research were derived from the genetically diverse progenitor stock of 'Kraków Super Strain' (KSS), maintained as non-overlapping generations on a 16L:8D photoperiod, at 30±1°C and 60±5%RH, with *ad libitum* fodder under these standard conditions every four weeks. Fodder was nine parts organic flour to one part yeast, and covered with oats to provide traction to adults.

2.2 Establishment of experimental evolution lines; thermal regimes

Experimentally evolved populations were created in 2010 from the KSS, an outbred 30°C progenitor stock (Dickinson, 2018). Each was the product of 7 days of mating and oviposition between 50 males and 50 females, which were randomly selected and sexually mature. Following adult removal, the egg-laden fodder was then either transferred to 300 ml containers to develop at their ancestral temperature of 30±1°C (n= 20) or in simulated warming conditions of 38±1°C and 60±5%RH (n= 20), adhering to standard sampling recommendations (Bretman et al., 2024). Both thermal regimes were maintained using A.B. Newlife 75 Mk4 egg incubators (A.B. Incubators, Suffolk). The long-term 38°C treatment was not considered a heatwave as i) 35°C to 38°C is the CT_{opt} for several life history traits reported in *T. castaneum* (reviewed in Sales 2019), and ii) kept constantly since this regime was initiated ~14 years ago. From generation one, these independent replicate populations were continually maintained in their divergent thermal regimes; adults were separated from offspring through non-overlapping generations. At each generation, a random selection of 100 adults (~50:50 sex ratios) were paired for 7-days after which the parents were removed, eggs oviposited during this 7-day period, developed to seed the subsequent generation that took a period of ~30–35 under respective standard rearing conditions.

2.3 Experiment 1: General potential for heatwave tolerance (adaptation protocol)

To test whether male reproductive fitness could adapt to local thermal regimes, male pupae were isolated from randomly selected populations grown at control 30°C (n= 8 populations), or warm 38°C (n= 8 populations), conditions for 25 generations. 20 male pupae from each population were selected and stored in population-specific Petri dishes, to sexually mature over 12 days. Storage temperatures matched that of the males' original regime. Adult males were allocated to 30°C or 38°C treatments, or a 42°C 5-day heatwave simulation (section 2.4), in a fully factorial design (Fig. 1a) (following Hoffmann and Sgrò, 2011). Males were rested for one day at 30°C before their reproductive fitness was assessed (section 2.5) (n≈ 5 per population per treatment). See Fig. 1a for sample sizes, predictions, and interpretation.

2.4 Heatwave simulations and survival assessment

Previous studies investigating heat-stress vary widely with exposure times and intensities (reviewed in Hoffman et al., 2003; Walsh et al., 2019). Here, 12±2-day post-eclosion virgin males were exposed to heatwave simulations using Octagon 20 Eco incubators (Brinsea Incubation Specialists, Somerset) (in experiment 1 and 2). These heatwaves elevated air temperatures to 42°C for 5 days (also see, Sales et al., 2018; 2021). This treatment corresponds with a common heatwave event definition (Frich et al., 2002). Moreover, 42°C exceeds the CT_{opt} for several of *T. castaneum*'s life history traits by 5–7°C (Sales et al., 2018; Sokoloff, 1974). Furthermore, 42°C is an ecologically relevant temperature, with over 90 countries recently recording heatwave air temperatures above 41°C (Mherrera, 2016; Table 2.3 Sales 2019). Once heatwave simulations and their controls were

accomplished, survival was assessed as the number of adults with, or without, co-ordinated locomotion (in experiment 2).

2.5 Mating and males' reproductive fitness

When assessing males' reproductive fitness following experimental treatments (in experiment 1 and 2), virgin males were randomly paired with virgin females in 4 ml vials containing 0.5 grams [g] of fodder for a 2-day mating opportunity. Using KSS females from the ancestral stock mitigated possible co-evolutionary thermal and compatibility confounds (following Dolgin et al., 2006). After these monogamous pairings, females were transferred to Petri dishes containing 7 g of fodder to oviposit across two independent 10-day blocks. After female removal (terminating oviposition), offspring developed, over 35 days into mature adults. Male reproductive fitness was quantified by the sum of adult offspring produced over the sum of two independent 10-day blocks (block 1: days 1–10 and block 2: days 11–20). Matings, oviposition and offspring development were all maintained at 30°C.

2.6 Testes dissections

Tribolium have paired testes, each pair contains six ellipsoid follicles and the volume of each follicle was calculated from its circumference by drawing an outline using a segmented line tool within ImageJ (Schneider et al., 2012; for method see, Sales et al., 2021). When adult males were dissected to measure testes volume (in experiment 2), dissections were in saline buffer (1% NaCl solution) under a Zeiss Discovery V.12 stereomicroscope at x20 magnification using a 0.10*0.06 mm-fine-tipped forceps on a subset of the sample (for further details, see Sales et al., 2021). Each image was acquired by translocating both testes to 30 µl of buffer to a cavity slide, sealing with a coverslip, and capturing images with a phase-contrast microscope. Dissected males' elytra (right side) were also measured to control for body size (see, Vasudeva et al., 2019).

2.7 Experiment 2: Elucidating acclimation versus evolved response

Further assays aimed to determine whether evolution and/or acclimation underpinned any potential thermal adaptation (section 2.3). After 45 generations of experimental evolution, the egg laden fodder of each population, produced from a 7-day-long mating opportunity between 100 adults per population, was split in half the amount (egg clutches take ~ 3–4 days to hatch, see detailed assays within Vasudeva et al., 2019). Half the amount of fodder (laden with fertilised eggs) from each population remained at its regime temperature, the other half was transplanted to the reciprocal regime temperature. As transplants occurred immediately after adult removal cohorts were mostly unhatched, freshly oviposited egg clutches, which maximised the potential for developmental acclimation to novel temperatures (Zhang et al., 2015). Thus, four thermal ancestry-development combinations were produced (30°C-30°C, 30°C-38°C, 38°C-30°C, 38°C-38°C) (Fig. 1b). 20 male pupae from each ancestry-development temperature combination were selected and stored for 12 days in population-specific Petri dishes to sexually mature. Storage temperatures

matched that of the males' development. Testes volumes were measured on a subset of the sample prior to heatwave exposure (section 2.6). After exposure to a 42°C heatwave treatment (section 2.4), male survival (section 2.4), and reproductive fitness (section 2.5) were assessed ($n \approx 4$ per population per treatment). See Fig. 1b for predictions and interpretation.

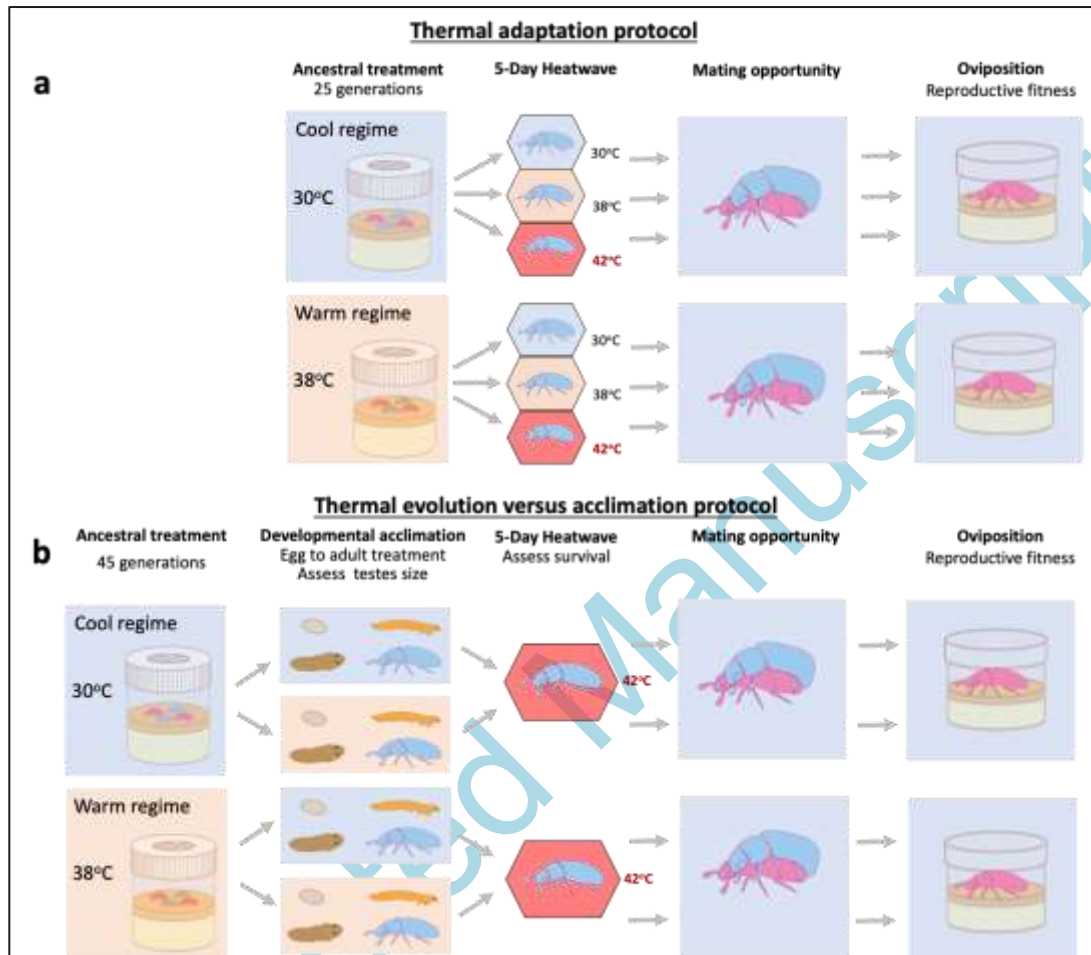


Fig. 1. Protocols for assessing exposures to ancestral versus novel temperatures on male reproductive biology

a. Experiment 1: Adaptation protocol in replicate populations ($n = 8$ independent populations per regime) cultured for 25 generations in thermal regimes 8°C apart. Males' (blue) reproductive fitness was assessed following differential temperature exposures for 5-days ($n \approx 5$ per population per treatment). Any local thermal adaptation present could be due to genetic evolution across generations and/or developmental acclimation via plasticity within the immediate generation

b. Experiment 2: Acclimation protocol at generation 45 to test the 30°C and 38°C regimes. The immediate generation of each thermal regime was either maintained at their historic temperature (30°C) for a single generation or reciprocally transplanted at the egg stage (sampled within 3 days, before egg hatching into larvae). Once developed into adults males testes were measured ($n \approx 4$ per population per treatment), and post heatwave survival ($n \approx 20$ per population per treatment) and reproductive fitness assessed ($n \approx 4$ per population per treatment). Evolutionary adaptation would be apparent if males from the warm 38°C regime (orange ancestry) performed relatively better following exposure to heatwaves. Conversely, acclimatory adaptation would be evident if males from the warm developmental temperatures (orange development) performed relatively better following heatwaves.

2.8 Statistical analyses

Data was analysed in RStudio (version 2022.07.2+576) with R (version 4.2.1.) (The RStudio Team, 2016). Figures consist of boxplots and error-bars. Boxplots display a mean dot, median line, Inter-quartile range boxes, 1.5*IQR whiskers and replicate data-points. Error-bars show means \pm standard error (S.E.). Significance thresholds are indicated by asterisks (***, $p < 0.001$; **, $p < 0.010$; *, $p < 0.050$), with letters identifying differences between treatments. All test statistics are reported to one decimal place (d.p.), all parameter estimates to one d.p., and all p values to three d.p. Percentage changes are relative to 30°C treatments. Generalised linear models (GLMs) were implemented with 'glm{stats}' (The R Core Team, 2017a), and Generalised Linear Mixed Models (GLMMs) with 'glmer{lme4}' (Bates et al., 2016). The most appropriate structures were identified with diagnostic residual plots (Barnard et al., 2011; Bolker et al., 2008; Field, 2009) using 'plot{graphics}' (The R Core Team, 2017b) and 'mcp.fnc{LMERConvenienceFunctions}' (Tremblay 2015). R^2 metrics were derived from a pseudo- R^2 function (Thomas et al., 2015; p.110), and from 'r.squaredGLMM{MuMIn}' (Barton, 2016) for GLMs and GLMMs, respectively. The overall statistical significance of a factor was calculated by analysis of deviance using 'drop1{stats}' (Thomas, et al., 2015). Post-hoc p values were calculated by 'summary{model}' (Crawley, 2013) for differences between factor levels and the intercept and by 'lsmeans{lsmeans}' (Lenth, 2017) was for pairwise Tukey-adjusted comparisons between all treatments.

The post-heatwave 20-day reproductive fitness of males from the 30°C and 38°C regimes in experiment 1 (Fig. 1a), produced count data, which were fitted with a log-linked Poisson GLMM. Fixed factors included the long-term thermal selection regime (30°C or 38°C), the 5-day male heatwave treatment (30°C, 38°C or 42°C), and the interaction between them. Both thermal regimes consisted of eight independently-evolved populations, which were represented by replication (5.7 ± 2.0 S.D. males). Therefore, to account for the population structure of repeatedly sampling from

independently evolving population within thermal regimes (Grueber et al., 2011; Crawley 2013, *p.*712), a random factor of population code (1-16) was included. Overdispersion was present, where variance of the data was greater than expected (usually due to zero-inflation, excessive variance and/or outliers), which risked unreliable parameter estimates. Therefore, a negative-binomial GLMM was fitted to the data with 'glm.nb{MASS}' (Thomas et al., 2015; Ripley et al., 2016), but despite including 'glm.nb(optimizer="bobyqa", optCtrl=list(maxfun=100000))' (Bolker et al., 2008; Powell, 2009) model convergence failed. Consequently, overdispersion was alleviated by adding a 'random observer level effect' to the original Poisson GLMM, where each individual replicate was coded as a different number (Bolker et al., 2008; Harrison 2014).

For the post-heatwave mortality analysis across ancestral and developmental treatments in experiment 2 (Fig. 1b), data-points were represented by the survival of males grouped into Petri dishes (19.8 ± 0.4 S.D. males). The proportion surviving was analysed with a logit-linked binomial GLM (Crawley, 2013; Thomas et al., 2015), where the response was a paired 'cbind(success, fail){base}' frequency variable, with success as survivors and fail as deaths (Thomas et al., 2015). The long-term selection regime (30°C or 38°C), developmental temperature (30°C or 38°C), and their interaction were inputted as fixed factors. Survival for each population was assessed by measuring a single group of individuals; therefore, no population-level pseudo-replication was present and enabling a GLM. A second logit-linked binomial GLM compared the evolved treatments to a baseline of progenitor KSS stock's survival at 30°C ($n = 17$ groups), the KSS being genetically diverse and 30°C-adapted.

The post-heatwave reproductive fitness of males across ancestral and developmental treatments in experiment 2 (Fig. 1b), was analysed with a log-linked Poisson GLMM, with the long-term selection regime (30°C or 38°C), developmental temperature (30°C or 38°C), and their interaction entered as fixed factors. A random factor of population code (1-15) accounted for replicated representation (4.3 ± 1.2 S.D. males) of the independently evolving populations. Both the 30°C and 38°C regimes contained eight populations, however, one 30°C population was omitted because no adults survived the 38°C-developmental-42°C-heatwave treatment. The initial Poisson GLMM was overdispersed. Overdispersion was not improved by fitting a negative-binomial GLMM, but was by adding a random observer level effect.

Male testes volume across ancestral and developmental treatments, in experiment 2, was analysed with an identity-linked Gaussian GLMM, with the long-term regime (30°C or 38°C), developmental temperature (30°C or 38°C), and their interaction entered as fixed factors. Elytra length was included as a co-variate. A random factor of population code (1-16) accounted for replicated representation (4.0 ± 0.0 S.D. males) of the independently evolving populations.

3. Results

General model structures and results are summarised in Table 1, while Supplementary Table 1 provides further detail with descriptive statistics, beta estimates, associated z/t scores and p values (Table S1).

3.1 Experiment 1: Reproductive fitness of males from warm-regime populations was more heatwave-tolerant

The mean 20-day reproductive fitness of males pooled from both the 30°C and 38°C selection regime differed following 30°C, 38°C and 42°C 5-day heatwaves ($\chi^2_{(2,273)} = 22.6$, $p < 0.001$; Fig. 2). In particular, the average (pooled) offspring counts were 213.6 ± 16.2 , 233.2 ± 13.7 and 159.1 ± 26.5 for 30°C, 38°C and 42°C, respectively. However, the mean male reproductive fitness from each thermal regime across the three 5-day heatwave temperatures did not differ ($\chi^2_{(1,274)} = 0.7$, $p = 0.388$); 30°C-regime males produced 196.7 ± 24.0 offspring, while 38°C-regime males sired 205.4 ± 21.1 .

Nonetheless, there was a significant interaction as the regimes responded differently across heatwave temperatures ($\chi^2_{(2,273)} = 14.1$, $p < 0.001$). The reproductive fitness of males from both thermal regimes were comparable following 30°C ($z = 1.0$, $p = 0.923$) and 38°C heatwaves ($z = 1.1$, $p = 0.899$). However, the average reproductive fitness of 38°C-regime males was 55% greater following 42°C heatwaves relative to 30°C-regime males ($z = 3.5$, $p = 0.005$), indicating adaption to extreme temperatures. The reproductive fitness of males from the 30°C regime, after a 42°C heatwave, was also significantly reduced compared to 30°C ($z = -5.1$, $p < 0.001$) and 38°C ($z = -5.3$, $p < 0.001$) exposures. Whereas, the reproductive fitness of 38°C-regime males was relatively constant across heatwave-exposures.

3.2 Experiment 2: Fitness outcome was modulated by interaction of inter-generational evolution and developmental acclimation temperatures

Survival of adult males after a 42°C heatwave event was affected by the ancestral temperature of populations; relative to 30°C-regime males, 38°C males were 77% more resistant to heatwave mortality ($\chi^2_{(1,30)} = 83.1$, $p < 0.001$; Fig. 3). Moreover, the survival of 38°C-regime males in heatwaves was comparable to males from the 30°C-adapted progenitor KSS stock in standard 30°C conditions ($\chi^2_{(2,46)} = 155.4$, $p < 0.001$; $z = 1.1$, $p = 0.502$). The developmental temperature of the immediate generation was also important for survival, with populations developing at 38°C being 26% worse at surviving heatwaves than those developing at 30°C ($\chi^2_{(1,30)} = 23.6$, $p < 0.001$). Additionally, there was an interaction between ancestral temperature and developmental temperature on heatwave

survival ($\chi^2_{(1,30)} = 15.4, p < 0.001$). 30°C-regime male heatwave survival was lower than 38°C males at 30°C ($z = -7.6, p < 0.001$), and at 38°C ($z = -4.3, p < 0.001$), but was relatively consistent across developmental temperature ($z = 1.4, p = 0.519$). During this assay, one of the populations from 30°C failed to survive at 38°C, so was lost within this set-up (therefore, $N=7$ as opposed to $N=8$ populations). Contrarily, 38°C-regime survival was greatest when the immediate generation grew at 30°C ($z = 5.5, p < 0.001$).

The 20-day reproductive fitness of adult males after a 42°C heatwave was affected by the ancestral temperature of populations. Relative to the 30°C regime, 38°C populations were 41% more productive ($\chi^2_{(1,126)} = 4.0, p = 0.047$; Fig. 4), indicating local thermal adaptation. Additionally, the immediate generation's developmental temperature altered reproductive fitness. Males from populations developing at 38°C sired 29% fewer offspring than those at 30°C ($\chi^2_{(1,126)} = 7.5, p = 0.006$). There was no interaction between developmental and ancestral temperature on reproductive fitness ($\chi^2_{(1,126)} = 0.7, p = 0.393$). There was also no difference in reproductive fitness across developmental differences within the 30°C-regime ($z = 2.4, p = 0.076$) and 38°C-regime males ($z = 1.6, p = 0.391$). Likewise, reproductive fitness was similar between the ancestral regimes within the 30°C ($z = -1.2, p = 0.639$) and 38°C ($z = -2.2, p = 0.133$) developmental temperatures. However, the reproductive fitness of 30°C-regime males developing at 38°C was significantly lower than the 38°C regime developing at 30°C ($z = -3.4, p = 0.003$).

Overall, adult male testes volume was not affected by their evolutionary thermal regime ($\chi^2_{(1,126)} = 0.7, p = 0.401$), their immediate developmental temperature ($\chi^2_{(1,126)} = 0.8, p = 0.373$), or their body size ($\chi^2_{(1,126)} = 0.0, p = 0.868$). However, there was an antagonistic interaction of evolutionary regime and developmental temperature on testes volume ($\chi^2_{(1,126)} = 40.4, p < 0.001$, Fig. 5), which was relatively smaller in populations developing in temperatures discordant from their ancestry. Specifically, males from the cool 30°C regime had relatively smaller testes if they developed 38°C ($z = -3.8, p = 0.001$), whereas males from the warm 38°C regime had relatively smaller testes if they developed 30°C ($z = -5.5, p = 0.001$).

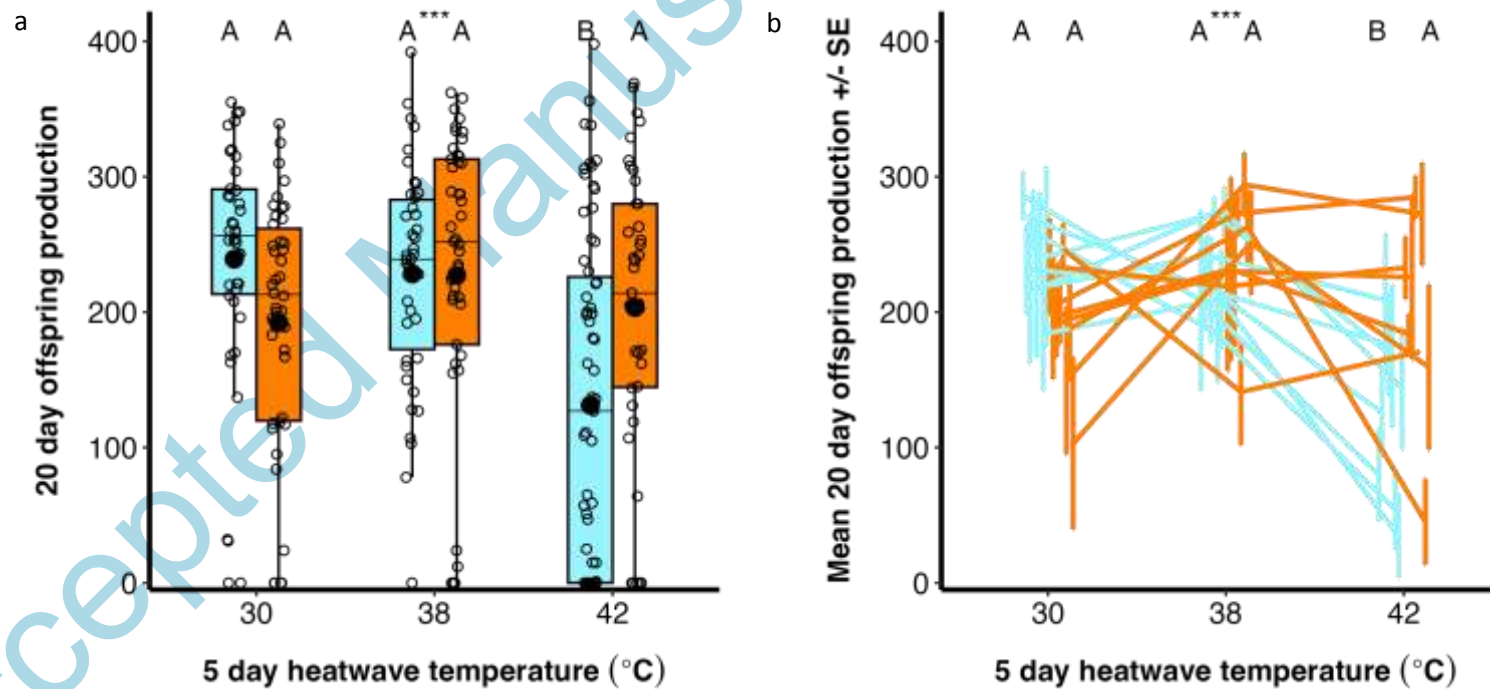


Fig. 2. Comparison of male reproductive fitness from independent populations under different thermal regimes after exposure to a range of 5-day heatwave temperatures. **A**, Contrast between relative warm 38°C regime males (orange fill) ($n_{\text{population}} = 8$) and 30°C regime males (blue fill) ($n_{\text{population}} = 8$), where each population was replicated (5.7 ± 2.0 S.D. males). **B**, Population-specific patterns across heatwave temperatures, sample sizes and colour scheme match (a).

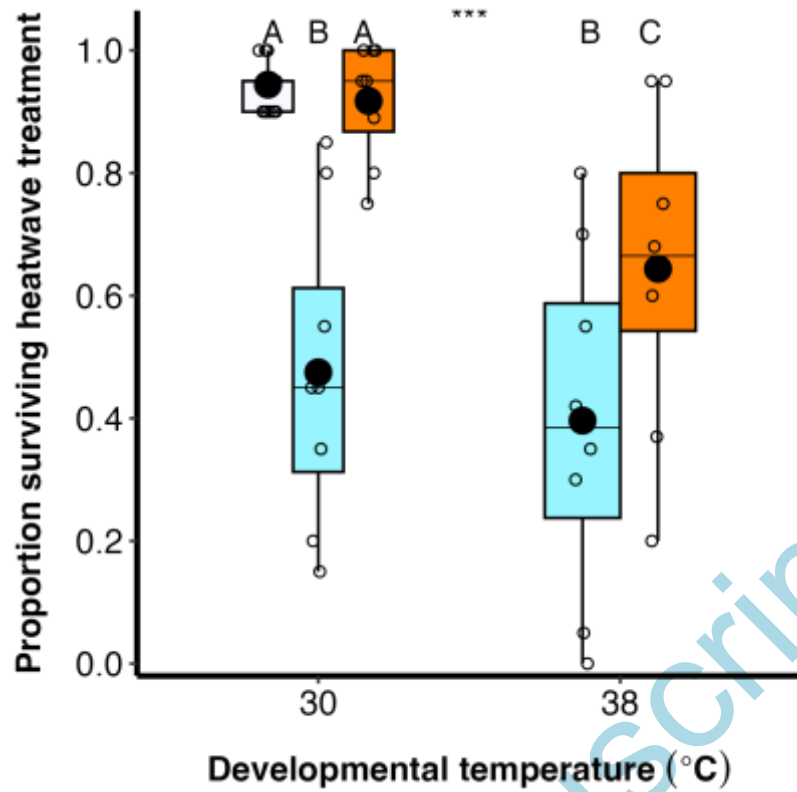


Fig. 3. Comparison of male post-heatwave survival between populations maintained at 30°C or 38°C. Contrast between relatively warm 38°C regime males (orange fill) ($n_{\text{population}} = 8$) and 30°C males (blue fill) ($n_{\text{population}} = 8$) survival following a 42°C 5-day heatwave. Comparison to males from 30°C-adapted progenitor KSS stock, maintained at 30°C, and not exposed to heatwaves (white fill) ($n = 17$). Each data-point was a group (19.8 ± 0.4 S.D. males) maintained in single-sex groups.

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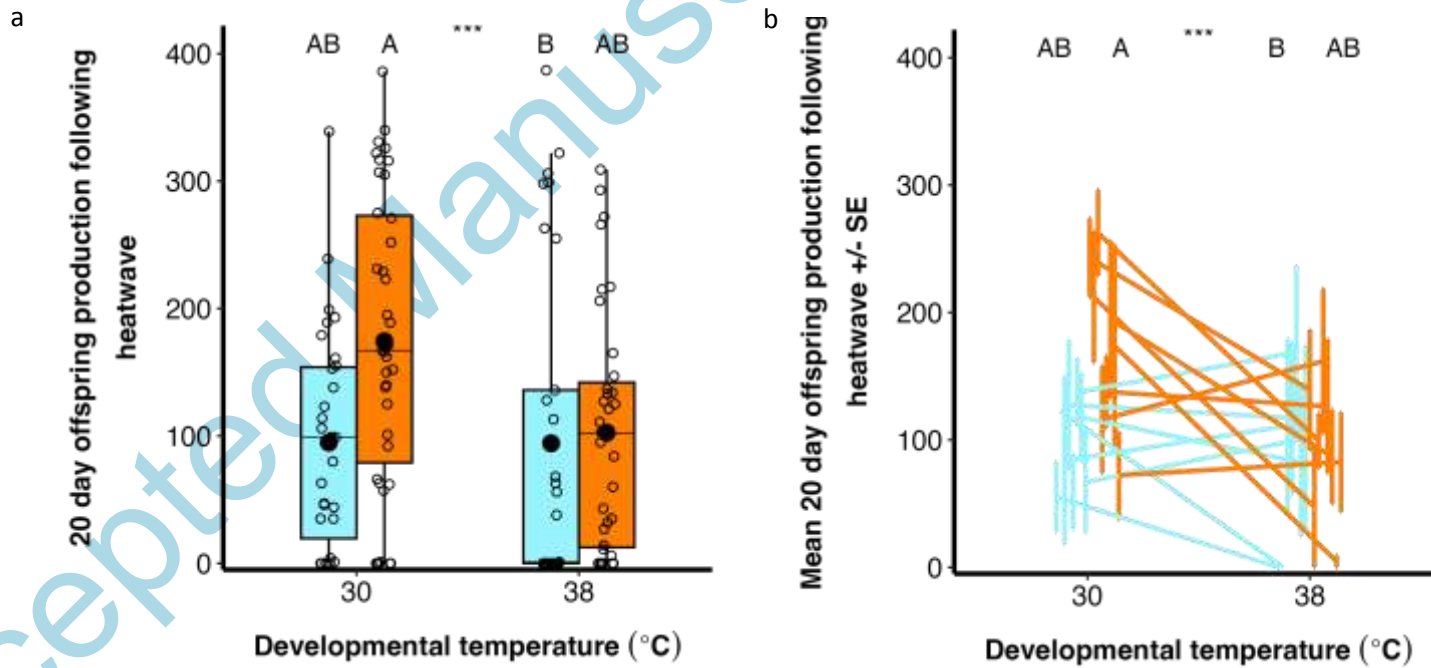


Fig. 4. Comparison of ancestral or developmental temperature impacts on male reproductive fitness after exposure to a 5-day heatwave. **A**, Contrast between relatively warm 38°C-regime males (orange fill) ($n_{\text{population}} = 8$) and 30°C-regime males (blue fill) ($n_{\text{population}} = 7$), where each population was replicated (4.3 ± 1.2 S.D. males). **B**, Population-specific patterns across developmental temperatures, sample sizes and colour scheme match (a).

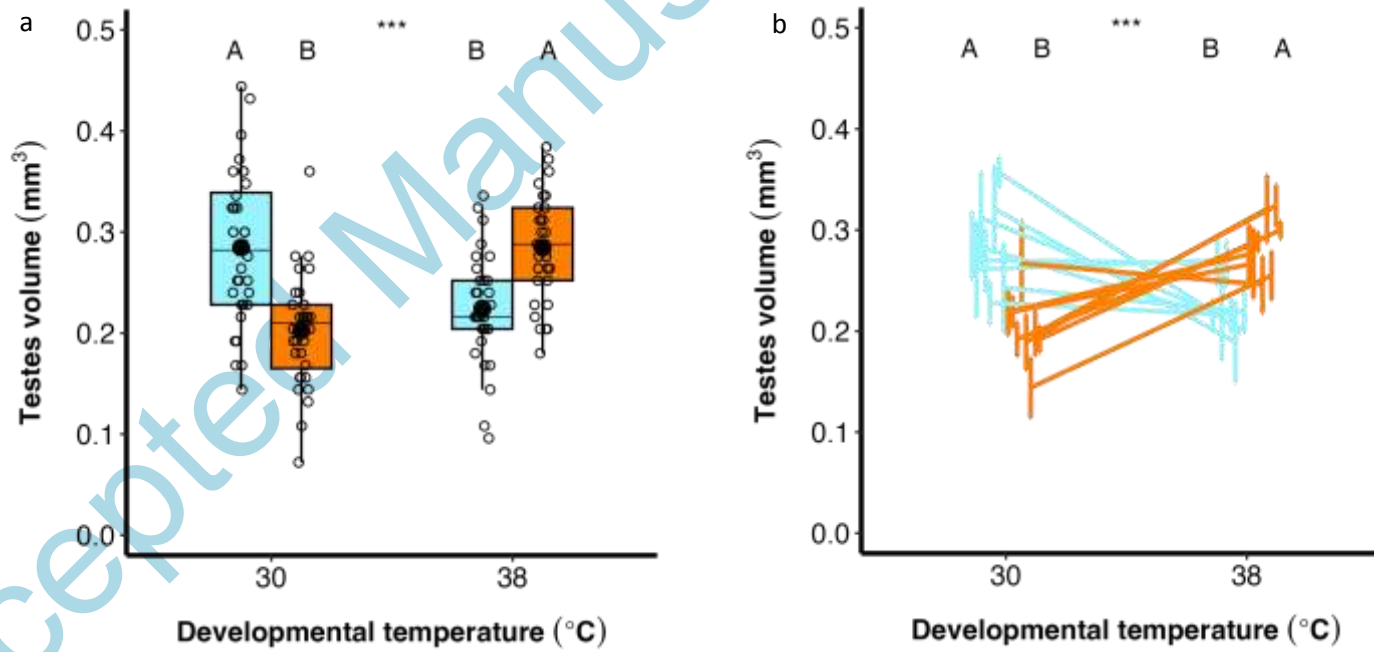


Fig. 5. Comparison of ancestral or developmental temperature impacts on testes volume. **A**, Contrast between relatively warm 38°C regime males (orange) ($n_{\text{population}} = 8$) and 30°C males (blue) ($n_{\text{population}} = 8$), where each population was replicated ($4 \pm 0\text{S.D.}$ males). **B**, Population-specific patterns across developmental temperatures, sample sizes and colour scheme match (a).

Table 1. Model summaries for experiments testing whether potential adaptation to increasing mean temperatures improves males' tolerance to heatwave extremes in the red flour beetle (*T. castaneum*). Descriptive statistics, model parameters, and R packages are reported in Supplementary Table 1.

Response variable	Experiment number	Fixed factor	DF	χ^2	<i>P</i>	Model, distribution and link function	error	Random effects	R ²
Male survival after 42°C heatwave	2	Ancestral regime temperature	1	83.1	<0.001	GLM Binomial (logit)		NA, only one replicate per population (percentage survival in a group)	43%
		Developmental acclimation temperature	1	23.6	<0.001				
		Regime*development	1	15.4	<0.001				
		Residual	28						
Male reproductive fitness	1	Ancestral regime temperature	1	0.7	0.388	GLMM Poisson (log)		Hierarchical population nesting to prevent pseudo-replication Random observer effect to control for overdispersion	m14% c99%
		Heatwave temperature	2	22.6	<0.001				
		Regime*heatwave	2	14.1	<0.001				
		Residual	271						
Male reproductive fitness after 42°C heatwave	2	Ancestral regime temperature	1	4	0.047	GLMM Poisson (log)		Hierarchical population nesting to prevent pseudo-replication Random observer effect to control for overdispersion	m12% c99%
		Developmental acclimation temperature	1	7.5	0.006				
		Regime*development	1	0.7	0.393				
		Residual	125						
Male testes volume (mm ³)	2	Ancestral regime temperature	1	0.7	0.401	GLMM (identity)	Gaussian	Hierarchical population nesting to prevent pseudo-replication Elytra length was a co-variate to control for body-size	m27% c27%
		Developmental acclimation temperature	1	0.8	0.373				
		Regime*development	1	40.7	<0.001				
		Elytra length	1	0.0	0.868				
		Residual	123						

4. Discussion

4.1. Local thermal adaptation and heatwave resistance of male fertility

Heatwaves reduced the reproductive fitness of males from experimentally-evolved 30°C-regime populations by approximately half, which conformed to previous experiments (Sales et al., 2018; Vasudeva et al., 2021). Elevating populations by 8°C for 25 generations raised the CT_{opt}/CT_{max} for male fertility, resulting in a 55% relative increase in post-heatwave reproductive fitness. The consistent improvement across independent population replicates suggested adaptation rather than genetic drift (Garland and Rose, 2009). Moreover, this warm adaptation may have been a conservative estimate because post-heatwave recovery, oviposition, and offspring development occurred at 30°C; because of logistical constraints and to preserve experimental consistency between treatments (Dolgin et al., 2006), which was likely advantageous for developing offspring from 30°C-regime males.

38°C-regime males were predicted to perform relatively worse at 30°C because adaptation to increasing temperature seems physiologically difficult (Li et al., 2015), and can trade-off against cold resistance (David et al., 2005). Surprisingly, here in *T. castaneum* this was not apparent. Previous work investigating thermal adaptation of *Tribolium* species is limited, especially for male fertility. However, *T. castaneum*, which is more tropical in its distribution than *T. confusum* and *T. madens*, has higher CT_{opt}/CT_{rng} for survival and population fitness in culture comparison experiments (Freeman, 1962; Howe, 1956; Park and Frank, 1948; Raros and Chiang, 1970). Here, thermal selection on 38°C-regime males has elevated their fertility CT_{opt} beyond their ancestral CT_{opt} for fertility and other life-history traits including: larval survival (Sokoloff 1974), oviposition behaviour (Waterhouse et al., 1971), population fitness (Howe 1956; Sokoloff 1974), and female fertility (Sales et al., 2018).

There is a greater amount of published research outside *Tribolium* for exploring local thermal adaptation using *Drosophila*, focusing mainly on survival traits (n= 18 studies, 6 species) (Table 4 Hoffmann et al., 2003). However, some studies have shown local thermal adaptation of male fertility, like here in *T. castaneum*, by using: a) inter-specific comparisons, b) intra-specific contrasts, or c) experimental evolution. Generally, the inter-species CT_{rng} for male reproductive fitness, was between 6-32°C (n= 9 species) (Table 1 David et al., 2005), and correlated with latitude. For example, heat-tolerant tropical species *Zaprionus indianus* (Araripe et al., 2004) was fertile at 30°C, while the European *D. subobscura* was unculturable beyond 25°C (Moreteau et al., 1997). Similarly, CT_{max} for male fertility seemed to correlate with latitude intra-specifically, such as with *D. melanogaster* (n= 24 populations) (Rohmer et al., 2004). CT_{max} for *D. melanogaster* male fertility has been artificially selected from 29°C to 32°C, but no higher (Chakir et al., 2002; Zatssepina et al., 2001). Beyond *Tribolium* and *Drosophila*, research demonstrating local thermal reproductive adaptation seems infrequent but includes: nematodes (*Caenorhabditis* spp.) (Harvey and Viney, 2007; Prasad et al., 2011), cattle (*Bos* spp.) (Rahman et al., 2018;

Skinner and Louw, 1966), butterflies (Karlsson and Wiklund, 2005), and aphids (Fig. 2 Buckley and Kingsolver 2012).

4.2. Potential physiological mechanisms for thermal adaptation of male fertility

The underlying resistance of warm-adapted male fertility to heatwaves may be due to CT_{max} increasing for spermatogenic traits. Post-heatwave sperm from 38°C-regime males could have been relatively more viable, motile and/or less prone to breaks. Sperm motility and viability was shown to underlie CT_{max} patterns for reproductive fitness in *D. melanogaster* and *D. simulans* (Fig. 4, Chakir et al., 2002), the more tropical *D. melanogaster*'s motility and fertility was 1.5°C higher comparatively. Other research has linked reduced sperm length in *Drosophila* species to increased heat resistance, because of the decreased likelihood of breaks (Araípe et al., 2004; David et al., 2005). Likewise, warm-adapted males' spermatogenesis may more consistently produce morphologically-normal sperm at higher temperatures (Rohmer et al., 2004).

38°C-regime males may also have more general adaptations to mitigate heatwave damage to general body condition. Behaviourally, they may have more sensitive thermotaxis to limit heat-exposure (Amos et al., 1968; Kim et al., 2015). Morphologically, they may have developed better circulation and insulation to thermoregulate testes (Skinner and Louw 1966), and/or less permeable cuticles to limit water stress (Noh et al., 2016). Metabolically, they may have developed more heat-stable and efficient proteins like phosphoglucose isomerase (PGI) (Dahlhoff et al., 2008). They may have better antioxidant defences in the testes to limit DNA damage (Kodama et al., 1997), and/or produce sperm membranes rich heat-tolerant polyunsaturated fatty acids (van Dooremalen et al., 2011). Local thermal adaptation, as seen here in *T. castaneum*, is likely linked to more efficient and effective heat shock protein (HSP) expression under extreme temperatures, which has been frequently reported in *Drosophila* (n= 20 studies) (Table 5, Hoffmann et al., 2003). First, several HSPs have been characterised in *T. castaneum*, and have been associated with traits like heat-shock survival (Mahroof et al., 2005c, 2005b; Schinko et al., 2012). Second, specific HSPs like *HSP70* and *HSP90*, are thought to protect and regulate spermatogenesis, as they are expressed comparatively strongly in the testes during stress in other species (Han et al., 2016; Long et al., 2015), and spermatogenic failures occur when the HSPs are interrupted (Dix et al., 1996).

4.3. Potential genetic mechanisms for thermal adaptation of male fertility

The 38°C-regime males' heatwave tolerance could be due to thermal selection on phenotypes suggested above, which have genetically-evolved through existing allelic diversity and mutations (Bijlsma and Loeschcke, 1997). Variability in genetic diversity for heat tolerance should exist, with CT_{max} differing between reproductively isolated populations (reviewed in Allen et al., 2006; Hoffmann et al., 2013). Other *T. castaneum* strains maintained in the same environment show some variability in thermotolerance, for

example, 42°C caused a 50% decline in reproductive fitness of KSS, 100% in GA-1, and 100% mortality in GFP (Dickinson, 2018; Sales et al., 2024).

In *Drosophila*, the variability of traits' thermotolerance has been related to specific genetic components including: genome size, chromosome number, inversion frequencies, single nucleotide polymorphism (SNP) mutations, and/or gene duplications (Balanya, 2006; Bundgaard and Barker, 2017; Gienapp et al., 2008; Vollmer et al., 2004). Generally, larger genomes and higher ploidy are thought to be better for adaptation, as there is more raw material for selection (Meirmans et al., 2006). However, with the relatively few generations of *T. castaneum* experimental evolution, genetic changes are likely smaller scale; SNPs and allele frequencies. Fertility-linked changes may focus on the Y-chromosome, which across *D. melanogaster* populations influenced 50% of the variation in CT_{max} for male fertility (Rohmer et al., 2004). Selection on 38°C-regime male genomes may have been purifying to remove deleterious alleles and/or positive for beneficial ones. Examples of thermal selection on alleles for fertility-linked genes in other species include: *fer-15* (Kenyon, 2011), *alg-3/4* (Plesnar-Bielak et al., 2017; Prasad et al., 2011), and the *dynein* gene family (Carvalho et al., 2001). Examples of thermal selection on alleles for traits linked to broader condition include: HSPs (Table 5 Hoffmann et al., 2003; Ramon et al., 2014), PGI (Dahlhoff et al., 2008), DNA repair (Paul et al., 2008), and mtDNA (Camus et al., 2017; Ellers et al., 2008).

4.4. Thermal adaptation by acclimation or evolution?

Experimental evolution to increased mean temperature proved adaptive for 42°C-heatwave resistance in *T. castaneum*; the survival and reproduction of 38°C-regime males, as the mean pooled across 30°C and 38°C acclimation temperatures, was greater than males from 30°C populations. However, unexpectedly, the post-heatwave survival and reproduction of 38°C-regime males was greatest when the developmental temperature of the immediate generation was 30°C rather than 38°C. Likewise, there was an antagonistic interaction between ancestral and developmental temperature for testes volume. These findings indicate that acclimation temperature is context specific for certain fitness traits.

Several species, including *D. melanogaster*, have shown that acclimating the parental generation, the immediate generation during development, or the immediate generation as adults over prolonged periods to warmer conditions can improve subsequent metabolism and survival (Gilchrist and Huey, 2001; Table 2 Hoffmann et al., 2003; Seebacher et al., 2015). Less frequently demonstrated is high temperature acclimation being beneficial for reproductive traits (Araripe et al., 2004; Fenkes et al., 2017; Harvey and Viney, 2007; Porcelli et al., 2017; Prasad et al., 2011; Purchase et al., 2010). Conversely, other research parallels the *T. castaneum* findings here, that acclimation to elevated temperature seems neutral, or even detrimental to fitness outcomes, in subsequent heat-stress. For example, studies of butterflies (Bauerfeind and Fischer, 2014), parasitoid wasps (Scott et al., 1997), and *Drosophila* spp. (Vollmer et al., 2004) found that increasing developmental temperature exerted life-history costs, without improving subsequent adult performance in heat-stress. Other studies have found that elevated acclimatory

temperatures impacted negatively on sperm traits (Adriaenssens et al., 2012; Ojima et al., 2015; Zeh et al., 2014).

Overall, there is uncertainty across literature as to whether inter-generational evolution or intra-generational acclimation is more important for adaptation, and whether acclimation is beneficial or detrimental (n= 64 articles) (Table 6.2, Sales, 2019). Furthermore, mixed fitness outcomes have been found for: a) different traits while keeping the species and acclimation method constant (Janowitz and Fischer, 2011; Porcelli et al., 2017; Stillwell and Fox, 2004; Woestmann and Saastamoinen, 2016), b) different acclimation methods while holding the species and traits constant (Kellermann et al., 2017; Porcelli et al., 2017; Richter et al., 2010; Scott et al., 1997; Zeh et al., 2014). The varied effectiveness of acclimation between studies, organisms, acclimation types and traits to could be due to several interacting causes (Boggs, 2016; Ellers et al., 2008; Hoffmann et al., 2003; Sgrò et al., 2016). In particular, fitness outcomes from acclimatory periods likely depend on how close the temperature is to a trait's CT_{max} , the timing of its onset, its duration, and the timing between acclimation and stress-testing.

Acclimation is thought to alter gene expression to create a more heat resistant phenotype through a variety of pathways including: metabolism (Ellers et al., 2008), nutrient transport (Veilleux et al., 2015); antioxidants (Kodama et al., 1997), solvent modifiers (Salvucci, 2000), lipid membranes (van Dooremalen et al., 2011) and HSPs (Mahroof et al., 2005b). The failure of the 38°C-developmental acclimation to benefit 38°C-regime males' heatwave resistance may have been because 38°C was too low to stimulate the correct triggers for an effective induced acclimatory response (Fig. 1 Tomanek 2008; Angilletta 2009). Alternatively, the potential adaptive mechanisms of 38°C-regime males may be genetically fixed with little scope for plasticity, or that the phenotypic changes required above CT_{opt} were too costly (Overgaard et al., 2011). Conversely, the acclimatory period may have been too protracted and/or hot so the costs of stress may have outweighed the benefits of altered gene expression (Stillwell and Fox, 2004; Terblanche et al., 2005). For example, 42°C heat-shocks on butterfly (*Junonia orithya*) pupae produced morphologically adaptive colouration associated with summer morphs, however, higher temperatures produced maladaptive colouration and mortality (Mahdi et al., 2011). Similarly, if the 38°C-acclimation of the 38°C-regime proved stressful, and despite their evolutionary genetic adaptation of heatwave tolerance, their CT_{opt} may still be closer to 30°C. Thus, their metabolism could have benefitted from a relaxation at 30°C, prior to heatwave challenge. The drain on reproductive performance with super-optimal development has also been demonstrated in other insects (Franke et al., 2014; Vollmer et al., 2004). There could have even been a hormetic effect, with the active reallocation of resources from reproduction to soma maintenance, if acclimation proved stressful (Chirault et al., 2015; Sheridan and Bickford, 2011).

4.5. Limitations and extensions

The baseline control temperature selected in the experiments was 30°C, which is the ancestral temperature, but marginally suboptimal for several life history traits (approximately 35°C), and it is likely that 38°C regime experienced the upper boundaries of

their CT_{opt} . Therefore, testing in a common garden intermediate temperature (e.g., sub-optimal for both regimes in opposing directions) might yield further insights and help better understand how individuals/populations respond to different thermal averages, which are likely under continued global climate change.

Across both experiments post-heatwave recovery, oviposition, and offspring development occurred at 30°C, which may have benefitted 30°C regime males and reduced effect sizes. Similarly, the adaptive reproductive fitness of 38°C regime males to higher temperatures may have been hindered by all female mates being from 30°C-KSS stock as opposed to the 38°C regime. For fuller understanding of potential local thermal adaptation at different levels of life history, differential temperature treatments could be applied on mating behaviour, fertilisation, and maternal effects. Moreover, experiment two only assessed survival and reproductive fitness after 42°C as this was the treatment most of interest, but equivalent controls at 30°C and 38°C would have been comprehensive.

Future work should also elucidate proximate mechanisms likely to promote adaptation and the role of gametes in this with signatures for adaptive plasticity. Here, the response of *T. castaneum* has been shown with constant temperature changes, however, 'Jensen's Inequality' predicts that traits respond differently with fluctuating conditions, including their magnitude and mean relative to CT_{opt} (Franke et al., 2014; Rolandi and Schilman, 2018; Zeh et al., 2014). Because of mixed findings in previous research, and the need for relevance to natural environments, further research into effects of temperature fluctuations has been recommended; so too has incorporating interactions between multiple realistic stressors like inbreeding (Pedersen et al., 2011) and dietary stress.

4.6. Conclusions Here, experimental evolution with warming could rapidly improve the heatwave resistance of male reproductive biology, but fitness outcomes seemed dependent on the interaction of evolution and acclimation. Finding that male fertility could adapt to long-term warming is broadly positive, especially in the context of previous findings that spermatogenesis can recover from heatwave damage (Sales et al., 2021, 2018, 2024). However, our work exploring thermal plasticity via hardening (Sales et al., 2021, 2018) or acclimation (here; Vasudeva et al., 2019), have shown that plasticity is only adaptive under certain specific thermal contexts (e.g., developmental exposure of gametes at lower temperatures, 30°C versus 38°C). In general, this makes studying fertility impacts to global climate change using ectothermic models exciting with the technological evolution of genomics, epigenomics and metabolomics (Kokko et al., 2017). Although the experimental designs here were balanced, several other contrasts could extend this framework, in addition to investigating many other response variables and treatment combinations available. Now that the experimental evolution has exceeded over 100 generations, divergence across cellular, morphological, and/or genomes potentially underlying male fertility seems possible and urgent.

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