Genetic and thermal variation influences adaptation to fluctuating temperature in the seed beetle, *Callosobruchus maculatus*

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Summary

The impacts of climate change on biological systems are notoriously difficult to measure, and laboratory studies often do not realistically represent natural fluctuations in environmental conditions. To date, most experimental studies of thermal adaptation test populations at constant temperatures, or they make incremental changes to an otherwise constant mean background state. To address this, we examined the long-term effects of stressful fluctuating daily temperature on several key life history traits in two laboratory populations of the seed beetle, *Callosobruchus maculatus*. These populations were kept for 19 generations at either a constant control temperature, T=29°C, or at a fluctuating daily cycle with T_{mean} = 33°C, T_{max}=40, and T_{min}=26. The latter being a simple representation of daily temperatures in southern central India in May, where this species originally evolved. We found that beetles that had evolved in stressful environments were smaller in body size when switched to a constant 29°C and had far greater reproductive fitness in comparison to beetles from both the constant control and continuously stressful 33°C environments. This suggests that beetles raised in environments with stressful fluctuating temperatures were more phenotypically plastic and had greater genetic variability than control treatment beetles and indicates that populations that experience fluctuations in temperature may be better able to respond to short-term changes in environmental conditions.

45 ***Introduction***

46 An increase in mean temperature negatively impacts a multitude of life history
47 traits in both plants (Hedhly *et al.*, 2009; Hatfield & Prueger, 2015) and animals
48 (Bauerfeind & Fischer, 2014; Berger *et al.*, 2017; Chen *et al.*, 2018; Lopez &
49 Adair, 2019; Ivimey-Cook *et al.*, 2021). In particular, heat stress caused by high
50 temperatures outside of a species' thermal optimum can disrupt reproduction in
51 mammals (Hansen, 2009) and fish (Lopez & Adair, 2019); inhibit growth and
52 development in plants (Hatfield & Prueger, 2015); and reduce longevity and
53 survival in insects (Berger *et al.*, 2017; Ivimey-Cook *et al.*, 2021).

54 Elevated temperatures, one of the main outcomes of rising anthropogenic
55 gas emissions (Lashof & Ahuja, 1990; Pereira *et al.*, 2012; Pachauri *et al.*, 2014),
56 can influence populations both directly, by increasing the rate of genome wide *de*
57 *novo* mutations (Berger *et al.*, 2017, 2021), or indirectly, by triggering
58 phenological mismatches between species and prey (Visser *et al.*, 1998, 2012;
59 see also Parmesan, 2006 for an extensive review). This increase in
60 environmental stress leads populations to either adapt to novel conditions through
61 phenotypic plasticity, to shift their geographic boundaries to match thermal
62 tolerance, or to go extinct (Holt, 1990; Pereira *et al.*, 2012; Hetem *et al.*, 2014;
63 Trisos *et al.*, 2020). Species in tropical habitats are predicted to be especially
64 vulnerable, as they have evolved far more narrow and specialized thermal ranges
65 (Janzen, 1967; Deutsch *et al.*, 2008; Walters *et al.*, 2012).

66 However, not only are the Earth's mean temperatures rising, but they are
67 also becoming more variable (Folguera *et al.*, 2011; Vasseur *et al.*, 2014;
68 Masson-Delmotte *et al.*, 2021). This increase in environmental variability is likely
69 to intensify the already detrimental impacts of elevated temperatures (Vasseur *et*
70 *al.*, 2014). Building on work by Deutsch *et al.* (2008), Vasseur *et al.* (2014) found
71 that changes to both the mean and variation in environmental temperature
72 produced long-term changes to population fitness. More specifically, performance

data collected from 38 globally distributed invertebrate species indicated that whilst increasing mean temperature had a mixture of both positive and negative effects on performance, which were largely latitude-dependent, increased variation was associated with more negative effects (Vasseur *et al.*, 2014). In addition, the authors highlighted the relative contribution of mean and variance in determining projected performance in the years 2050-2059. They found that a change in mean temperature explained only 32% of future species performance, whereas combining both the mean and variance simultaneously explained 93%. This highlights the importance of understanding how species react not only to changes in mean temperature, but also to changes in temperature *variation* in order to form accurate predictions of a population's response to climate change.

Studying the effects of climate warming and the resulting adaptive responses of organisms is often confined either to field studies, which normally consist of a multitude of uncontrollable ecological variables, or to laboratory studies, which have the benefit of increased experimental control but typically rely on the use of constant mean temperatures with very little consideration of natural fluctuations (Fischer *et al.*, 2011; Folguera *et al.*, 2011; Thompson *et al.*, 2013). As a result, populations could respond in an entirely different manner when exposed to fluctuating rather than constant thermal stress in the laboratory (Folguera *et al.*, 2011; Bauerfeind & Fischer, 2014). For example, reaction norms of the mosquito *Anopheles stephensi* under fluctuating thermal regimes were fundamentally different to those under constant temperatures (Paajimans *et al.,* 2013). Similarly, in the tropical butterfly *Bicyclus anyana*, life history traits were strongly influenced by both the mean and the amplitude of the experimental thermoperiod (Brakefield & Mazzotta, 1995). Indeed, the use of constant temperatures might significantly underestimate the impact of global warming on life history evolution (Fischer *et al.* 2011). These concerns clearly highlight the need to integrate more realistic thermal regimes into empirical studies (Niehaus *et*

101 *al.*, 2012; Paaijmans *et al.*, 2013) in order to fully understand how species are
102 responding to climate change (specifically increases in mean temperature and
103 increased variability) under more natural conditions (Folguera *et al.*, 2011; Colinet
104 *et al.*, 2015).

105 To this end, we investigated the long-term effects of thermal stress
106 caused by realistically fluctuating temperatures on the phenotypic plasticity and
107 adaptation of two laboratory populations of seed beetle, *Callosobruchus*
108 *maculatus*. Two different thermal regimes were used: in one regime, beetles were
109 kept at a constant control temperature of 29°C, and in the second regime we
110 exposed beetles to a more stressful and realistic fluctuating daily cycle with a
111 mean temperature of 33°C. This temperature regime was chosen because it is
112 representative of a May day in southern India, where the laboratory lines were
113 originally collected. The two beetle populations were then either kept in these two
114 environments for the entirety of the experiment or were *switched* to the other
115 environment for two generations without selection prior to assaying several key
116 life history traits (namely, development time, body mass and various measures of
117 fitness).

118 Previous work in the same species of beetle has highlighted the negative
119 effect of elevated temperatures on various life history traits (Rogell *et al.*, 2014;
120 Vasudeva *et al.*, 2014; Ivimey-Cook *et al.*, 2021). In addition, a series of papers in
121 by Hallsson and Björklund (2012) investigated how increasing temperature with
122 and without environmental fluctuation influenced reproductive success,
123 development time, and body size. Whilst predominantly focusing on the amount
124 of treatment-specific genetic and environmental variance for each trait, they found
125 that fluctuating environments had a significantly negative effect on fecundity but
126 little to no effect on hatching success, development time and body mass.
127 Importantly, our definition of fluctuating environments differs from theirs. Whilst

the term employed here represents daily sinusoidal fluctuations around a constant mean, representative of the natural environment that a seed beetle could experience, Hallsson and Björklund (2012) used the term to describe the addition of thermal noise around a linear increase from 30°C to 36°C. As such, we may expect entirely different life-history responses to higher mean temperatures that are also fluctuating around that mean.

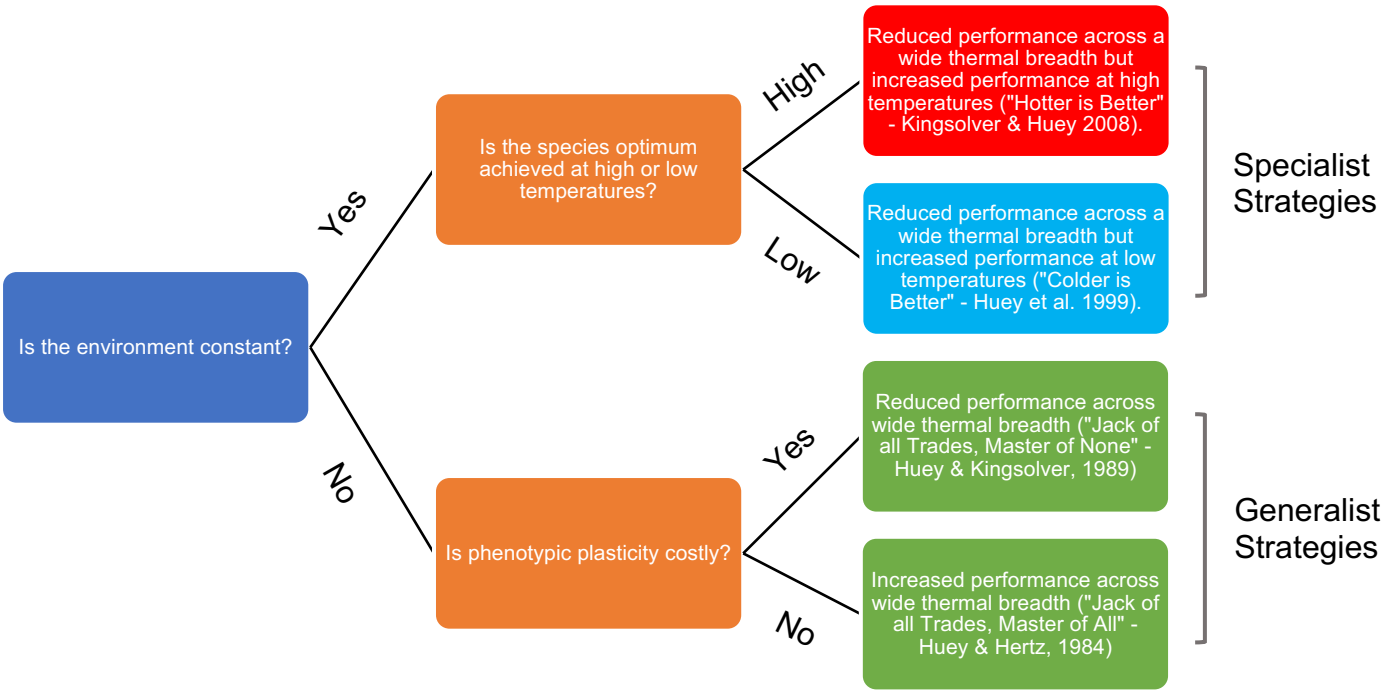
In particular, the heterogeneity of the environment an organism experiences, along with the associated costs and benefits of maintaining differing widths of thermal tolerance, can dramatically alter which genotypic strategies are favoured by selection. For instance, stabile and homogenous environments have been shown to lead to the evolution of specialists (Reboud & Bell, 1997) who reduce the width of their thermal niche in order to maximise performance. The particular direction of this specialization is largely dependent on the optimum temperature of the organism, be that hot: “Hotter is Better” hypothesis (Kingsolver & Huey, 2008, Fig. 1), or cold: “Colder is Better” hypothesis (Huey *et al.*, 1999, Fig. 1). Fluctuating environments may instead favour the evolution of generalists with increased phenotypic plasticity (or reversible phenotypic plasticity, Ratikainen & Kokko, 2019) and enhanced genetic variation (Reboud & Bell, 1997; see Table 1 and 2 from Kassen, 2002). However, this plasticity might carry a cost, for example if individuals trade off increased thermal tolerance with reduced reproductive performance (e.g. the “Jack of all Trades, Master of None” hypothesis - Huey & Kingsolver, 1989; Berger *et al.*, 2014; Fig. 1). Therefore, we would predict that populations evolving in fluctuating environments should underperform (i.e., through reduced fitness), in comparison to those in more constant benign environments. Alternatively, If phenotypic plasticity is instead beneficial and can evolve without a cost to reproduction, then beetles evolving in fluctuating environments should have higher performance (i.e. evolve to become “Jack of all Trades, Master of All” - Huey & Hertz, 1984 *or* “Superior Thermal

156 Generalists", see Berger *et al.*, 2014; Fig. 1) than those evolving in constant
157 benign environments. As a result, the relative comparison between benign and
158 stressful environments will largely depend on the costs and benefits of phenotypic
159 plasticity (see Fig. 1). Lastly, individuals from a benign environment that are then
160 *switched* to a stressful environment should suffer reduced reproductive
161 performance owing to a lack of evolved phenotypic plasticity.

162 The predictions for development time and body size were more
163 straightforward. Increases in temperature have been shown to reduce
164 development time in many insects (Hagstrum & Leach, 1973; Nylin & Gotthard,
165 1998; Hallsson & Björklund, 2012b; a; Czarnoleski *et al.*, 2013). Since the mean
166 temperature of the fluctuating environment was higher in our experiment, we
167 predicted that individuals that evolved in, or were later placed into, the fluctuating
168 environment would experience shorter development times. Finally, increasing
169 mean temperature whilst within fluctuating environments has been shown to
170 decrease body size in insects (Nylin & Gotthard, 1998; Kingsolver & Huey, 2008;
171 Hallsson & Björklund, 2012a; b; Czarnoleski *et al.*, 2013). Many organisms,
172 particularly insects, experience faster growth at higher temperatures; under these
173 conditions, decreasing cellular size and thus body size would allow for an
174 optimum rate of resource exchange (Czarnoleski *et al.*, 2013). Therefore, we
175 predicted that body size should be smaller in the populations that evolved in, or
176 were placed into, the fluctuating environment.

177 To investigate potential population-level differences we used two different
178 strains of *C. maculatus*, derived from USA and Leicester. Although we had no *a*
179 *priori* predictions about if and how any population-level differences would
180 manifest, previous research has highlighted the substantial genotypic and
181 phenotypic differences that exist between strains of *C. maculatus* (Rankin &
182 Arnqvist, 2008). Lastly, we also investigated relative differences between the two
183 sexes, as males and females in this species can vary in their response to

184 stressful environments (Stillwell & Fox, 2007; Rogell *et al.*, 2014). Previous
185 research on this species has shown that sexual dimorphism in traits such as body
186 size at emergence decreases when beetles are gradually exposed to novel,
187 higher temperatures over 18 generations (Hallsson & Björklund, 2012b; 2012a).
188 We therefore predicted that any differences between sexes would similarly
189 decrease in populations exposed to more stressful, fluctuating temperatures.
190



191

192 **Figure 1.** Hypotheses regarding reproductive performance and phenotypic plasticity
193 and the evolution of generalist or specialist strategies.
194

195 ***Methods***

196 *Study system*

197 *C. maculatus* is an agricultural pest that originates from Africa and Asia but is
198 currently found throughout the world. Adult seed beetles are facultatively aphagous –
199 that is, they do not require food or water to survive and acquire all the resources they
200 need during the larval stage within the bean. Aphagous adults can live up to two

weeks but adults with access to nutrients can live three weeks or more (Fox, 1993; Ursprung *et al.*, 2009). Females lay their eggs on the surface of dried legumes, such as mung beans (*Vigna radiata*) or black-eyed beans (*Vigna unguiculata*). Larvae from the fertilized eggs burrow into the bean and eclose as adults 23-27 days later. Females of this species start to mate and lay eggs as soon as they emerge (Beck & Blumer, 2014).

Laboratory evolution lines and climatic conditions

In our experiment, we used two different strains of *C. maculatus,* South India (SI) USA and SI Leicester, both derived from a population of beetles collected in Tirunelveli, India, in 1979 (Mitchell, 1991) and then subsequently raised at the University of Kentucky, USA, and the University of Leicester, UK (from 1992), respectively (Fricke & Arnqvist, 2004). We obtained these strains from Uppsala University in Sweden and maintained them at the American University of Paris for approximately 25 generations before establishing the replicates for the current experiment. Beetles were cultured exclusively on mung beans and kept in climate chambers at a constant 29°C, 50% relative humidity, and a 12:12h light:dark cycle. They were housed at aphagy in 1L jars with 250g of beans, and approximately 250-350 newly hatched beetles were transferred to new jars with fresh beans every 24 days on a continual basis.

For 19 generations prior to the experiment, we subdivided SI USA and SI Leicester populations (Fig. 2A) into four replicates each. Two “Control” replicates of each were maintained at a constant temperature of 29°C, while two “Stress” replicates were subject to daily temperature cycle (Fig. 2B). The daily cycle consists of 12 separate 2hr periods of constant temperature T_i,

$$T_i = T_{mean} + \Delta \mathrm{T} \sin\left(\frac{i-12}{12}\pi\right),$$

227 where T_{mean} = 33 °C, ΔT = 7°C, and i = 0,1…. 11. This is a stepwise sinusoidal
228 temperature cycle with T_{max}=40 and T_{min}=26 and which mimics typical late spring
229 condition in Southern India, where this species evolved. Humidity and light cycles
230 remained the same for all replicates: 50% relative humidity, and a 12:12 h light:dark
231 cycle.

232 Prior to the assays of body size, development time, and reproductive fitness (see
233 next section), we created two replicates of two populations (SI USA and SI Leicester;
234 see Fig. 2A/B) for:

235 1. beetles adapted to and assayed at Control conditions (**USA**-C and **LEI**-C);
236 2. beetles adapted (for one year) to and assayed in Stress conditions (**USA**-S
237 and **LEI**-S);
238 3. beetles adapted to Control conditions that were then acclimatized to Stress
239 conditions for two generations without selection prior to being assayed (**USA**-
240 C-to-S and **LEI**-C-to-S); and
241 4. beetles adapted to Stress conditions that were acclimatized to Control
242 conditions for two generations without selection prior to being assayed (**USA**-
243 S-to-C, **LEI**-S-to-C).

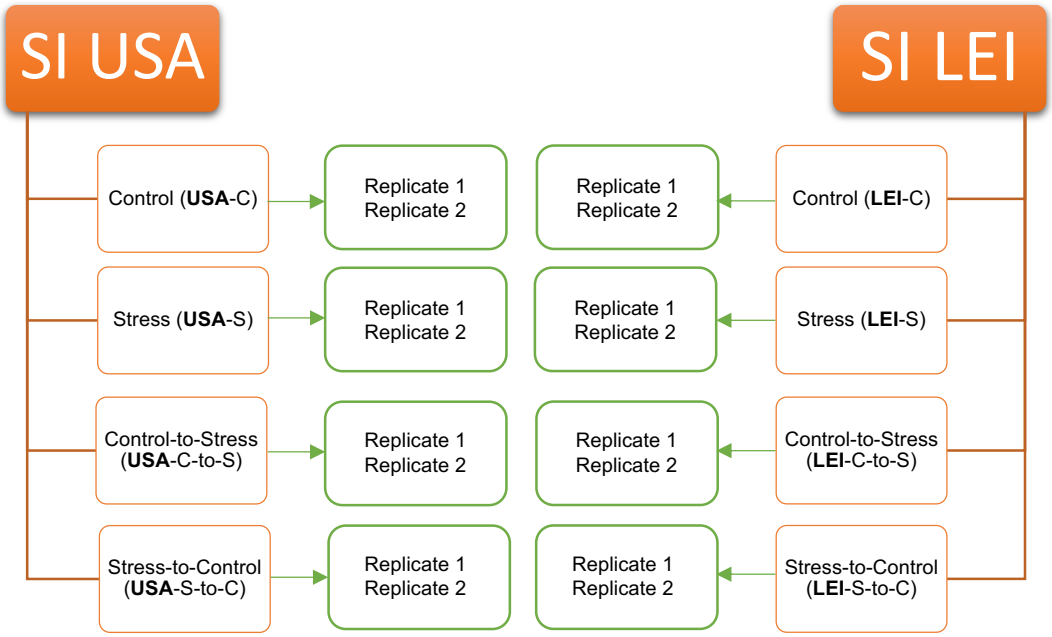
244

245 To separate genetic adaptation from phenotypic plasticity – that is, to control
246 for the influence of parental effects – we created "acclimatized" treatments by
247 allowing beetles from one set of conditions to adjust to the new set of conditions for
248 two generations prior to assays (C-to-S and S-to-C; Fig. 2B). Critically, to remove the
249 possible effects of natural selection and rule out the possibility that rapid genetic
250 responses might influence our measurements for the "acclimatized" treatments, we
251 randomly paired 50 pairs of virgin beetles during each of the two generations. That is,
252 we removed the effects of assortative mating.

253 For the Stress-to-Control (USA S-to-C, LEI S-to-C) and the Control-to-Stress
254 (USA C-to-S, LEI C-to-S) treatments, the following steps were performed. First, we

255 moved fertilized beans from jars into 48-well virgin chambers. When enough males
256 and females had hatched (from day 21-24 after hatch), we paired 60 males and 60
257 females in 60 60-mm petri dishes with 100 beans in each dish and placed these
258 dishes in the opposing chamber. We made sure that there were sufficient number of
259 beans in each dish for females to lay just one egg per bean, thereby eliminating
260 competition among multiple larvae within a bean. To further reduce larval
261 competition, we removed adult beetle pairs from the petri dishes after 72 hours.
262 After 19 days, we then choose the 50 petri dishes with the most fertilized
263 beans and put 48 fertilized beans from each petri dish into individual marked 48-well
264 virgin chambers. One day after eclosion, we randomly selected and paired 50
265 females and 50 males and put them into 50mm petri dishes with 80 beans. After 72
266 hours, the adult males and females were removed from the petri dishes. After 19
267 days, fertilized beans from each petri dish were transferred into 48-well virgin
268 chambers. We then marked the date of hatch and sex of all offspring and conducted
269 assays of development time, body mass, and reproductive fitness.
270 For the continuous C and S treatments, we skipped the acclimatization steps
271 and simply moved fertilized beans to virgin chambers prior to hatch, selecting one-
272 day old males and females for all subsequent assays.
273

A


SI USA
SI LEI
Control (**USA**-C)
Replicate 1
Replicate 2
Replicate 1
Replicate 2
Control (**LEI**-C)
Stress (**USA**-S)
Replicate 1
Replicate 2
Replicate 1
Replicate 2
Stress (**LEI**-S)
Control-to-Stress (**USA**-C-to-S)
Replicate 1
Replicate 2
Replicate 1
Replicate 2
Control-to-Stress (**LEI**-C-to-S)
Stress-to-Control (**USA**-S-to-C)
Replicate 1
Replicate 2
Replicate 1
Replicate 2
Stress-to-Control (**LEI**-S-to-C)

B

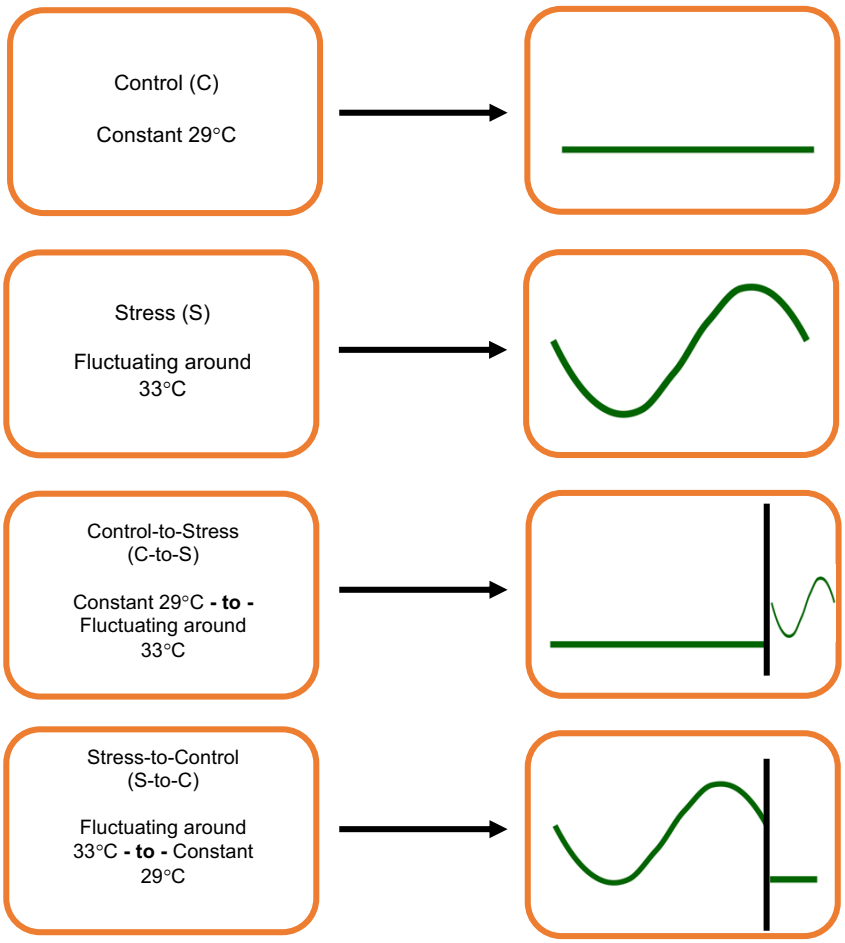

Control (C)
Constant 29°C
Stress (S)
Fluctuating around 33°C
Control-to-Stress (C-to-S)
Constant 29°C **- to -** Fluctuating around 33°C
Stress-to-Control (S-to-C)
Fluctuating around 33°C **- to -** Constant 29°C

Figure 2. (A) A schematic diagram of the four temperature treatments (Control, Stress, Control-to-Stress, and Stress-to-Control) across both the USA (SI USA) and Leicester (SI LEI) populations. (B) A representation of the four different temperature treatments used. The black bar in the bottom two treatments, denotes a switch from one treatment to another in order for acclimatization to occur over two generations prior to assaying.

Body mass assay

For each of the 16 populations, we weighed male and female beetles separately in two batches of 12 beetles each using an Ohaus Pioneer Plus Analytical Balance (Model PA214C). All virgin chambers were weighed empty, prior to adding the beetles to be weighed. We then calculated the difference between the weight of the virgin chamber before the beetles were placed inside and the weight of the virgin chamber after the beetles were placed inside. Beetles from the body mass assay were not used for the other assays.

Development time and fitness assays

Development time and fitness assays were done concurrently. We paired 50 1-day old male and female beetles and placed them into 60mm petri dishes filled with 85 beans. After 24 hours, each pair was moved together into a new 60mm petri dish filled with 75 beans, and the first dish was set aside in the same climate chamber. The next day, this procedure was repeated. The beetles were left in the third and final set of dishes until they died. The first dishes for each pair were marked “Day 0”, the second dishes were marked “Day 1”, and the third and final dishes were marked “Day 2+”. After 19 days for each dish, all the fertilized beans were placed into virgin chambers, which were monitored daily for the date of eclosion and the sex of all offspring. To calculate development time, we counted the number of days between the date that an egg was laid and the date the adult offspring eclosed. To calculate

LRS, we calculated the number of male and female offspring that emerged for each pair, from all three sets of dishes (Days 0, 1, and 2+). To calculate individual fitness (or λind), we took the dominant eigenvalue (using the popbio v. 2.7 package, (Stubben & Milligan, 2007) from an age-structured Leslie matrix (Leslie, 1945) where the top row denotes fertility and the sub-diagonal represents survival from age *t* to *t*+1. 24 days of development time were added prior to day 0-2+ fertility schedule.

Statistical analyses

All statistical analyses were performed in R v. 4.1.0 (R Core Team, 2016) using glmmTMB v. 1.0.2.9000 (Brooks *et al.*, 2017; Magnusson *et al.*, 2019) with pairwise interactions and slopes compared using emmeans v. 1.6.1 (Lenth *et al.*, 2019). Ggplot2 v. 3.3.4 (Wickham, 2009) was used for all graphical visualization. The overall effects of each variable were identified using the Type 3 Anova function from car v. 3.0-10 package (Fox *et al.*, 2012).

For both development time, body mass, and individual fitness, a Gaussian linear mixed effects model was fitted (after log-transforming development time to reduce right-skew) with the fixed effects of Population, Treatment, Sex (for development time and body mass), and all higher order interactions. In addition, the fixed factor of replicate number and, for development time, the random effect of parental ID was added to account for potential pseudoreplication. If the three-way interaction was non-significant (identified from the Anova function), it was removed in order to reduce model complexity. A generalized linear mixed effects model with Poisson distribution and similar fixed effect structure was fitted for LRS and age-specific reproduction, albeit without the variable Sex and all subsequent two/three-way interactions. In addition to a covariate of Day (and all higher-order interactions with Population and Treatment), a random effect of individual ID was added to the age-specific reproduction model to account for repeated measures. Both models were checked for overdispersion and zero-inflation (ZI) using DHARMa v. 0.4.1,

332 (Hartig, 2020) by simulating the residuals of a Poisson model. If significant, a variety
333 of error distributions with/without zero-inflated parameters (if ZI was identified) were
334 then fitted and the best model identified by Akaike's Information Criterion (AIC).
335

336 ***Results***

337 *Body mass*

338 Individual body mass was significantly affected by both of the interactions involving
339 Sex (Sex and Population: $\chi^2 = 25.95$, p<0.001; Sex and Treatment: $\chi^2 = 19.18$,
340 p<0.001; Table S1A) but not by the remaining two-way interaction of Population and
341 Treatment ($\chi^2 = 2.51$, p = 0.47) or the three-way interaction ($\chi^2 = 6.37$, p = 0.09).
342 Females were generally heavier than males (Fig. 3) in both populations. The
343 difference was more pronounced in the USA population (Average body mass (g): F-
344 LEI = 0.087, M-LEI = 0.075, estimate = 0.013, p<0.001; F-USA = 0.089, M-USA =
345 0.068, estimate = 0.021, p<0.001; Fig. 3, Tables S1A-B). The females of both
346 populations were similar in weight, but the USA males were smaller (F-LEI = 0.087,
347 F-USA = 0.089, estimate = -0.002, *p* = 0.35; M-LEI = 0.075, M-USA = 0.068,
348 estimate = 0.007, p<0.001; Fig. 3, Tables S1A-B). The relative difference between
349 male and female weight was largely dependent on temperature treatment (Fig. 3).
350 Overall, beetles raised in the S-to-C regime were smaller for both sexes in
351 comparison to other treatments (all comparisons significant to p = 0.05; Tables S1A-
352 B). However, the difference between male and female weights was far less
353 pronounced in the constant stress (S) treatment (albeit all differences were still
354 significant to p<0.001; F-S-to-C = 0.083, F-S = 0.089, F-C-to-S = 0.091, F-C = 0.089;
355 M-S-to-C = 0.064, M-S = 0.079, M-C-to-S = 0.071, M-C = 0.071; Tables S1A-B).

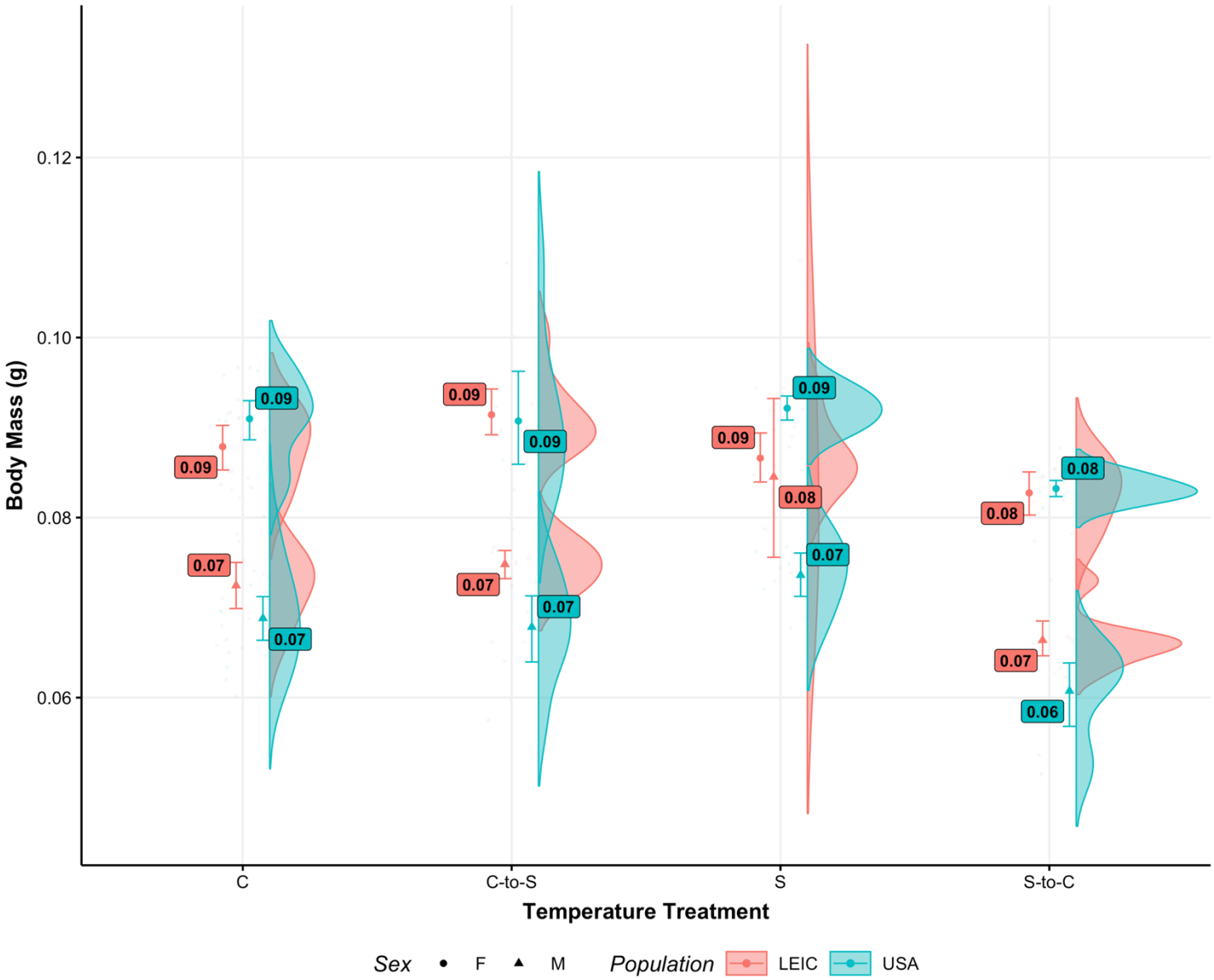


Figure 3. Average body mass of individuals from the Leicester (Red) or USA (Blue) populations in the C (Control), C-to-S (Control-to-Stress), S (Stress), or S-to-C (Stress-to-Control) temperature treatments. Sex is denoted with either circle (Female) or triangle (Male) points. Points represent mean values with accompanying standard errors. Half violins show the distribution of raw data.

Development time

Development time was influenced by both two-way interactions involving Temperature treatment (Treatment and Population: $\chi^2(3) = 105.94$, $p < 0.001$;

Treatment and Sex: χ^2 (3) = 11.76, *p* = 0.008). Similar to body mass, no detectable three-way interaction was found (χ^2 (3) = 1.71, p = 0.635). The USA and Leicester populations responded differently to the four temperature treatments, with beetles from the Leicester population developing faster on average. Populations from USA took significantly longer to develop in comparison to populations from Leicester in both the Control and S-to-C Treatments (Average Development Time (Days): LEI-C = 23.7, USA-C = 24.7, Ratio = 0.960, *p*<0.001; LEI-S-to-C = 23.1, USA-S-to-C = 24.1, Ratio = 0.961, *p*<0.001; Fig. 4) but not in C-to-S (LEI-C-to-S = 23.0, USA-C-to-S = 23.0, Ratio = 0.999, *p* = 1.00) or S Treatments (LEI-S = 22.5, USA-S = 22.3, Ratio = 1.01, *p* = 0.38; Fig. 4, Tables S2A-B). Despite these differences, both populations in the S treatment developed faster compared to other temperature treatments (LEI-S = 22.5, LEI-S-to-C = 23.1, LEI-C-to-S = 23.0, LEI-C = 23.7; USA-S = 22.3, USA-S-to-C = 24.1, USA-C-to-S = 23.0, USA-C = 24.7). In addition, whilst males typically developed faster in both populations (Fig. 4), only in the S-to-C treatment was this difference significant (F-S-to-C = 23.7, M-S-to-C = 23.50, Ratio = 1.008, *p*<0.001; Fig. 4, Tables S2A-B). However, both males and females in the S treatment developed faster than other temperature treatments and showed no sex difference in development time (F-S = 22.4, F-S-to-C = 23.7, F-C-to-S = 23.0, F-C = 24.2; M-S = 22.4, M-S-to-C = 23.5, M-C-to-S = 22.9, M-C = 24.1).

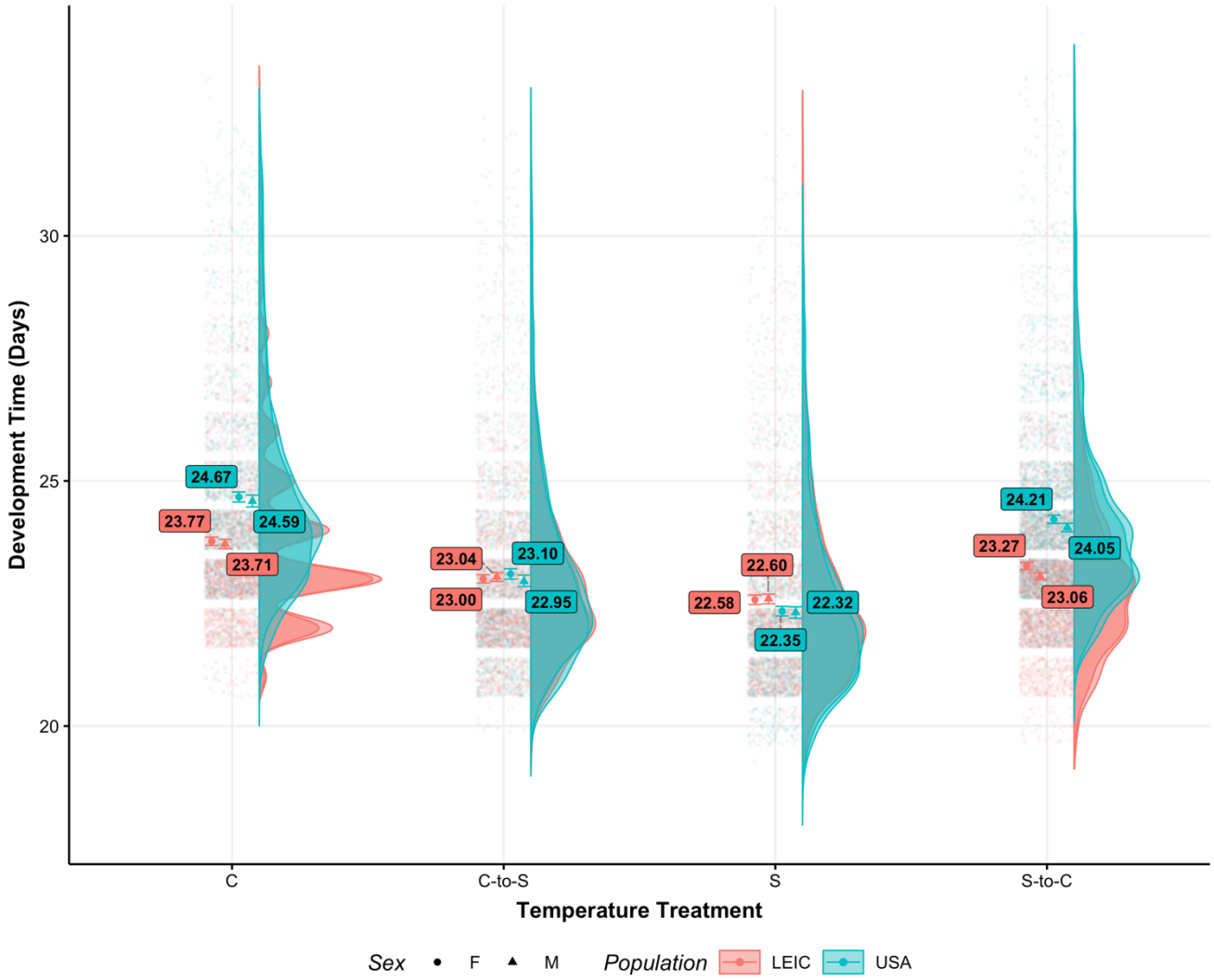
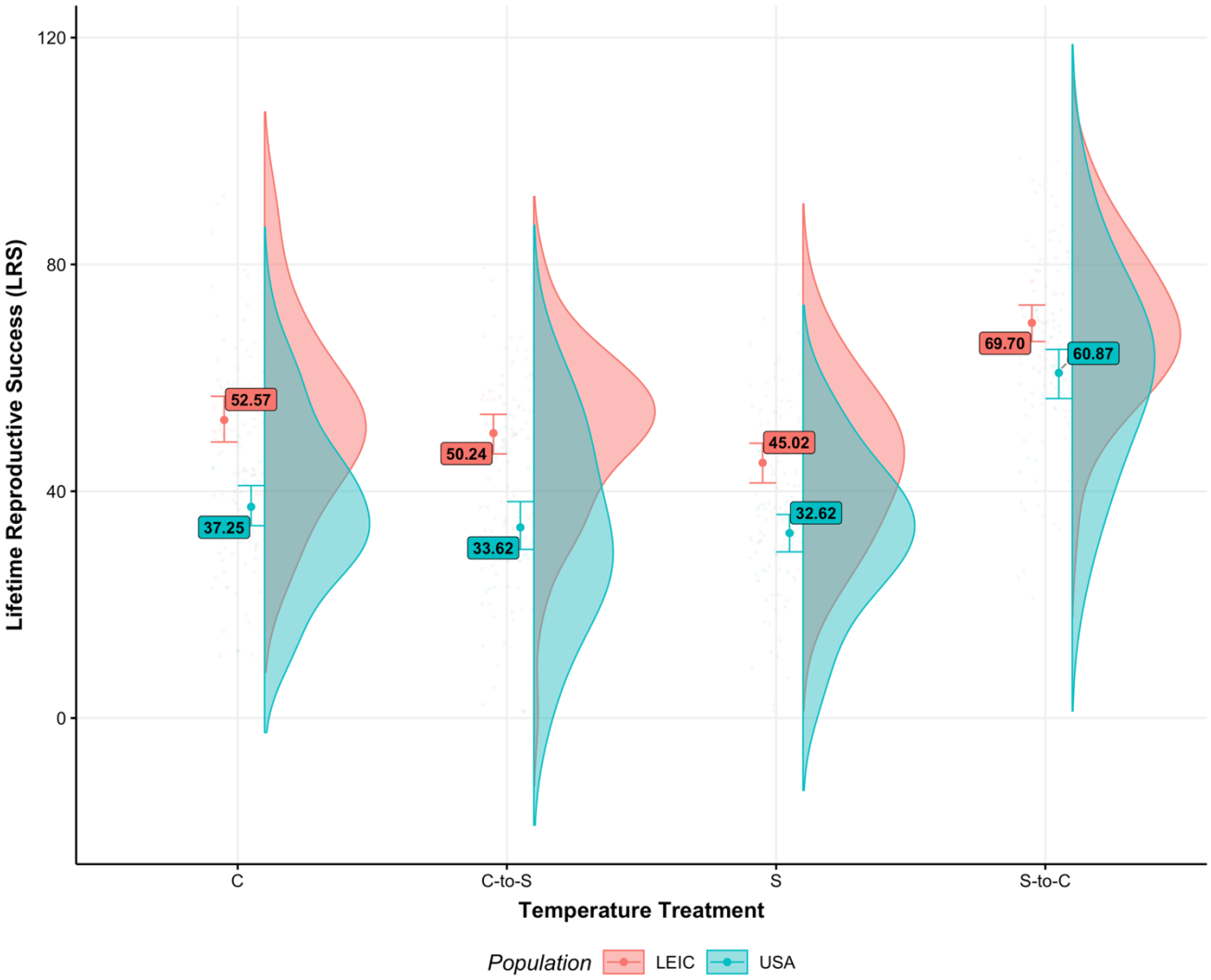


Figure 4. Average development time in days of individuals from the Leicester (Red) or USA (Blue) populations in the C (Control), C-to-S (Control-to-Stress), S (Stress), or S-to-C (Stress-to-Control) temperature treatments. Sex is denoted with either circle (Female) or triangle (Male) points. Points represent mean values with accompanying standard errors. Half violins with associated jittered points show the distribution of raw data.

Lifetime reproductive success

LRS was significantly influenced by the interaction between Population and Treatment (χ^2 (1) = 12.61, p = 0.005). Across all temperature treatments, LRS was

395 lower in the USA population than in the Leicester population (Average LRS: LEI =
396 53.2, USA = 40.2, Ratio = 1.32, $p<0.001$; Fig. 5, Tables S3A-B). The largest
397 differences between populations occurred in the C-to-S and C treatments (LEI-C-to-S
398 = 50.2, USA-C-to-S = 33.6, Ratio = 1.49, $p<0.001$; LEI-C = 52.6, USA-C = 37.2, Ratio
399 = 1.41, $p<0.001$; Fig. 5, Tables S3A-B). Despite these differences, LRS was highest
400 in the S-to-C treatment for both populations (LEI-C = 52.6, LEI-S-to-C = 69.7, Ratio =
401 0.75, $p<0.001$; USA-C = 37.2, USA-S-to-C = 60.9, Ratio = 0.61, $p<0.001$; Fig. 5,
402 Tables S3A-B).



403

404 **Figure 5.** Average lifetime reproductive success (LRS) of individuals from the
405 Leicester (Red) or USA (Blue) populations in the C (Control), C-to-S (Control-to-

Stress), S (Stress), or S-to-C (Stress-to-Control) temperature treatments. Points represent mean values with accompanying standard errors. Half violins show the distribution of raw data.

Age-specific reproduction

The interaction between population, treatment and day significantly influenced age-specific reproduction (χ^2 (3) = 203.54, p<0.001, Fig. 6). Whilst continuous C and S treatments exhibited similar age-specific patterns across both populations (Fig. 6), there were striking differences between S-to-C and C-to-S treatments. This was particularly apparent on Days 1 and 3 of reproduction (Fig. 6). Individuals from both LEI-S-to-C and LEI-C-to-S treatments exhibited far greater Day 1 reproduction than all other treatments from both populations (including the analogous S-to-C and C-to-S treatments). In stark contrast, USA-S-to-C exhibited increased reproduction *after* Day 1, with the result that Day 3 of reproduction was comparable with the Day 1 reproduction for LEI S-to-C (Fig. 6). This variation in reproductive schedule resulted in large differences in age-specific slopes between the USA S-to-C and LEI S-to-C treatments (Average change in average offspring produced per day: LEI S-to-C = -7.14, USA S-to-C = 8.78, estimate = -15.92, p<0.001; Fig. 6, Tables S4A-B).

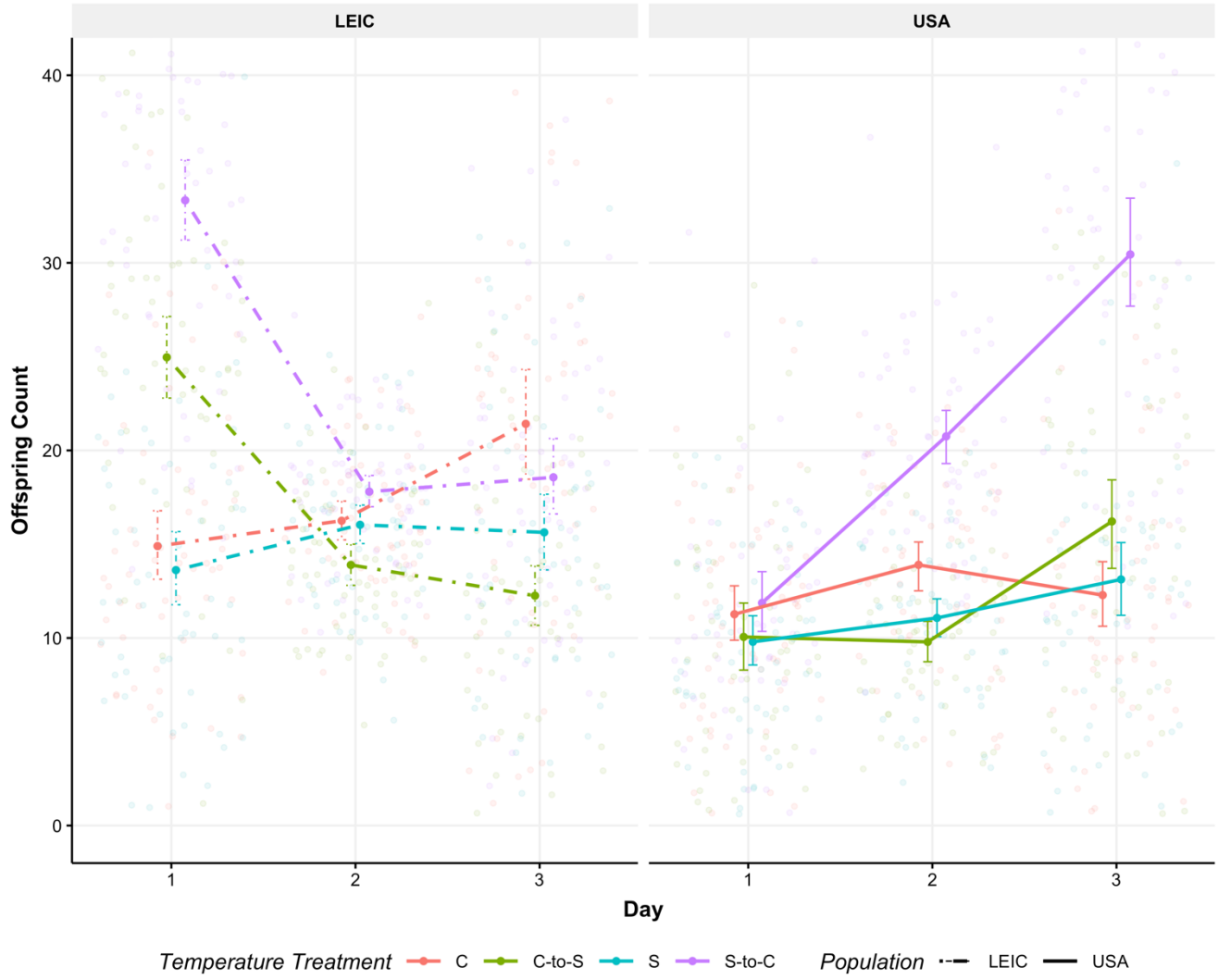
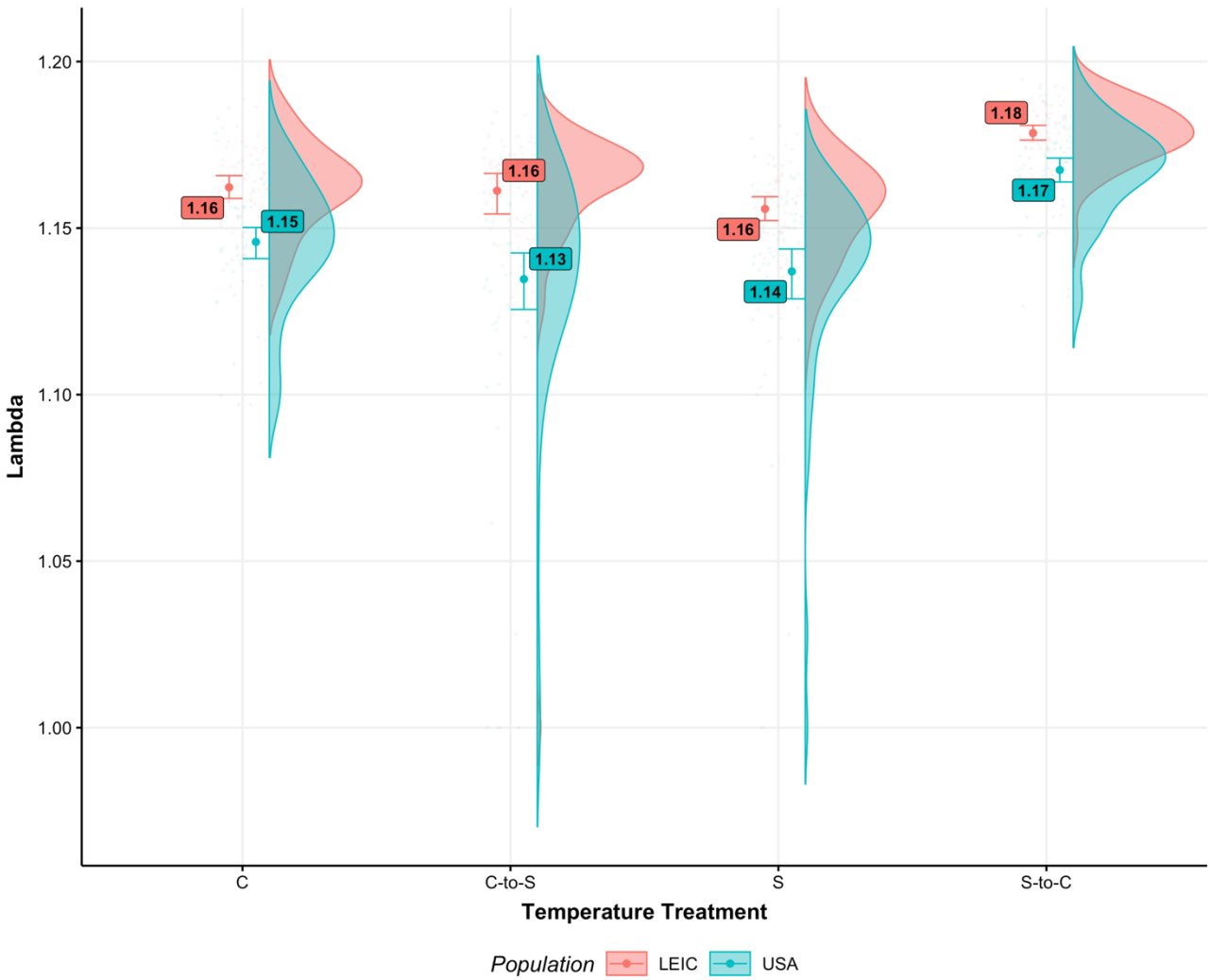


Figure 6. Age-specific reproduction (LRS) of individuals from the Leicester (dash-dot) or USA (solid) populations in the C (Control, Red), C-to-S (Control-to-Stress, Green), S (Stress, Blue), or S-to-C (Stress-to-Control, Purple) temperature treatments. Points with error bars represent mean values with accompanying standard errors. Jittered points represent raw data.

Individual fitness

Whilst the individual main effects of Population and Temperature treatment significantly influenced λind (χ^2 (1) = 81.02, p <0.001, χ^2 (3) = 111.23, p <0.001,

434 respectively; Fig. 7), the interaction between these two did not (χ^2 (3) = 7.64, *p* =
435 0.054). In a similar manner to LRS, the USA population had reduced λind over all
436 temperature treatments (Average λind: LEI = 1.164, USA = 1.146, estimate = 0.018,
437 p<0.001; Fig. 7, Tables S5A-B). Across both populations, individuals from the S-to-C
438 treatment had the highest λind (C = 1.154, C-to-S = 1.148, S = 1.146, S-to-C =
439 1.173, all comparisons p<0.001; Tables S5A-B). This was also consistent when
440 looking within each population (all comparisons p<0.001).
441



442 **Figure 7.** Fitness (Lambda) of individuals from the Leicester (Red) or USA (Blue)
443 populations in the C (Control), C-to-S (Control-to-Stress), S (Stress), or S-to-C

(Stress-to-Control) temperature treatments. Points represent mean values with accompanying standard errors. Half violins show the distribution of raw data.

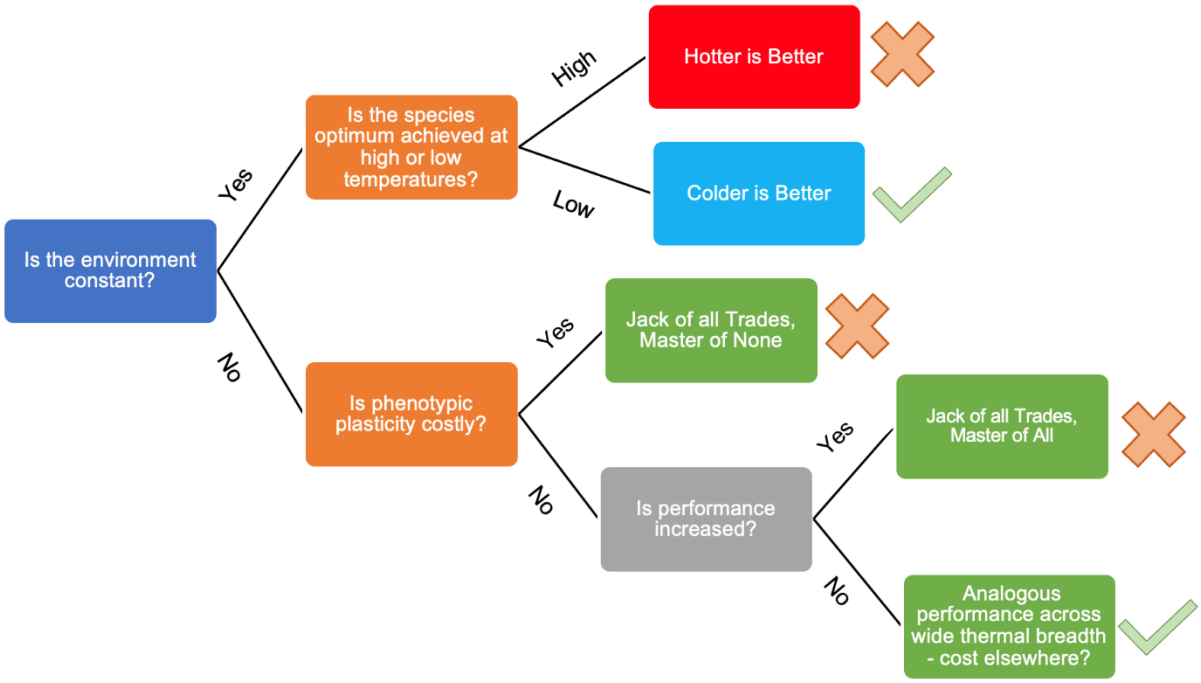


Figure 8. Hypotheses and subsequent results (indicated with ticks and crosses) regarding reproductive performance and phenotypic plasticity and the evolution of generalist or specialist strategies.

Discussion

Previous hypotheses (Lewontin, 1974; Nevo, 1978) have suggested that evolution in fluctuating environments should select for genetically variable individuals that are robust to a wide range of thermal conditions, often termed generalists (Kassen, 2002). In this experiment, generalists exposed to novel benign conditions (S-to-C, Fig. 8) (although not those exposed to continuously stressful environments, S, see below) had higher reproductive fitness (higher LRS and λind) than specialists that were exposed to novel stressful conditions (C-to-S) or kept continuously in a benign environment (C). These results are broadly in line with previous research conducted

in the opportunistic bacterial pathogen, *Serratia marcescens* (Ketola *et al.*, 2013). Heterogenous environments selected for bacterial clones that had faster growth and higher yield than those grown under constant environments, even when introduced into the same control temperature of 31°C (Ketola *et al.*, 2013). This increased growth may be due to far greater selective pressure to remove mutations that could otherwise accumulate in more constant benign environments (Ketola *et al.*, 2013). However, results from a digital evolution experiment have suggested that this may only allow for optimization in short-term rather than long-term evolvability (Canino-Koning *et al.*, 2019).

We found that individuals within stressful fluctuating environments evolved strategies similar to thermal-tolerance generalists as well as performance specialists (Gilchrist, 1995). More specifically, individuals were able to tolerate and survive at wide thermal breadth, presumably owing to the evolution of phenotypic plasticity, but they reached maximal performance and experienced increased LRS only when conditions were optimal (Gilchrist, 1995; Ketola *et al.*, 2013). This could in part be due to differential gene expression as a result of exposure to a novel thermal environment (also known as cryptic genetic variability, McGuigan & Sgro, 2009; Paaby & Rockman, 2014). Similarly, this release of cryptic genetic variation could explain why individuals that were predicted to perform poorly when switching from a benign control to stressful fluctuating environment (C-to-S), suffered no fitness cost. However, the relative merits of this form of variation have been queried and require additional empirical testing (McGuigan & Sgro, 2009; Edwards & Yang, 2021; Johansson *et al.*, 2021). Regardless, these results suggest that the evolution of phenotypic plasticity in experiment did not carry substantial fitness costs.

When comparing body mass across treatments, individuals from the S-to-C group were significantly smaller (across both populations and sexes), despite having similar development times to those of the constant control group. Smaller body size could therefore be a cost of adaptation (Kassen, 2002; Ketola *et al.*, 2013) to the

488 fluctuating conditions that is only detected in the benign environment and is
489 manifested in reduced body size (albeit with no negative effects on fitness). However,
490 whether this reduction in mass is a disadvantage is difficult to determine. Large size
491 provides a fitness advantage due to sexual selection (Andersson & Iwasa, 1996),
492 whereas small size decreases larval competition (Messina, 2004). Interestingly,
493 similar to Hallsson & Björklund (2012a), we found that sexual dimorphism in adult
494 body size decreased in the continuously stressful environment (S), although this
495 could be considered population-specific as individuals from USA failed to show such
496 a decrease.

497 Lastly, there were consistent population-level differences across all
498 environmental treatments. In general, beetles from the USA population produced
499 fewer offspring, took longer to develop (although this difference disappeared under
500 stressful conditions), and had greater sexual dimorphism in body size compared to
501 individuals from the Leicester population. Such genotypic variation between
502 populations of the same species (particularly in *Callosobruchus maculatus*) has been
503 found to impact larval competition (Messina, 1991), various morphometric measures
504 (Rankin & Arnqvist, 2008), egg-to-adult survival and offspring production (Rankin &
505 Arnqvist, 2008), and adult longevity and mortality (Fox *et al.*, 2004a; b). However, in
506 this experiment, one particular observation stood out. Whilst a switch from a stressful
507 environment to a benign regime (S-to-C) was associated with an increase in LRS and
508 λind in both USA and Leicester populations, there was a noticeable difference in the
509 age-specific reproductive schedules between the USA and Leicester populations.
510 Leicester beetles produced more offspring on the first day and fewer on days two and
511 three, whilst in the USA population the opposite was observed. Why such a
512 difference in reproductive schedule exists deserves deeper investigation. If genetic
513 variance differed between populations, this may explain why Leicester individuals
514 were able to respond more quickly, with greater Day 1 reproduction. This could also
515 in part explain why development time was similar across all experimental

temperature regimes for the Leicester population. Together this clearly highlights the benefits of comparing and contrasting results from multiple strains, as the response of species to environmental change can often be highly population-specific.

Successfully mimicking natural conditions within the laboratory is key to understanding how species will react to climate change. As temperatures increase and become more variable, the incorporation of more realistic conditions into laboratory studies such as these will allow us not only to predict more accurately how wild species will respond to climate change, specifically thermal stress, but also to produce accurate species- and population-specific thermal reaction norms (Paaijmans *et al.*, 2013). This study highlights the need to include both constant and more variable conditions in laboratory studies and suggests that the evolution of generalist and specialist strategies will be vital in explaining species-specific responses to climate change.

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References

Andersson, M. & Iwasa, Y. 1996. Sexual selection. *Trends Ecol. Evol.* **11**: 53–58. Elsevier.

Bauerfeind, S.S. & Fischer, K. 2014. Simulating climate change: temperature extremes but not means diminish performance in a widespread butterfly. *Popul. Ecol.* **56**: 239–250.

Beck, C.W. & Blumer, L.S. 2014. A handbook on bean beetles, Callosobruchus maculatus. *Natl. Sci. Found. URL Httpwww Beanbeetles Orghandbook Pdf Last Accessed 16 June 2015*.

Berger, D., St\aangberg, J., Baur, J. & Walters, R.J. 2021. Elevated temperature increases genome-wide selection on de novo mutations. *Proc. R. Soc. B* **288**: 20203094. The Royal Society.

Berger, D., Stångberg, J., Grieshop, K., Martinossi-Allibert, I. & Arnqvist, G. 2017. Temperature effects on life-history trade-offs, germline maintenance and mutation rate under simulated climate warming. *Proc. R. Soc. B Biol. Sci.* **284**: 20171721. Royal Society.

Berger, D., Walters, R.J. & Blanckenhorn, W.U. 2014. Experimental evolution for generalists and specialists reveals multivariate genetic constraints on thermal reaction norms. *J. Evol. Biol.* **27**: 1975–1989.

Brakefield, P.M. & Mazzotta, V. 1995. Matching field and laboratory environments: effects of neglecting daily temperature variation on insect reaction norms. *J. Evol. Biol.* **8**: 559–573.

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., *et al.* 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R J.*, doi: 10.32614/rj-2017-066.

Canino-Koning, R., Wiser, M.J. & Ofria, C. 2019. Fluctuating environments select for short-term phenotypic variation leading to long-term exploration. *PLOS Comput. Biol.* **15**: e1006445. Public Library of Science.

Chen, H., Zheng, X., Luo, M., Guo, J., Solangi, G.S., Wan, F., *et al.* 2018. Effect of short-term high-temperature exposure on the life history parameters of Ophraella communa.

Colinet, H., Sinclair, B.J., Vernon, P. & Renault, D. 2015. Insects in Fluctuating Thermal Environments. *Annu. Rev. Entomol.* **60**: 123–140.

Czarnoleski, M., Cooper, B.S., Kierat, J. & Angilletta, M.J., Jr. 2013. Flies developed small bodies and small cells in warm and in thermally fluctuating environments. *J. Exp. Biol.* **216**: 2896–2901.

Deutsch, C.A., Tewksbury, J.J., Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C., *et al.* 2008. Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* **105**: 6668–6672.

Edwards, C.B. & Yang, L.H. 2021. Evolved Phenological Cueing Strategies Show Variable Responses to Climate Change. *Am. Nat.* **197**: E1–E16. The University of Chicago Press.

Fischer, K., Kölzow, N., Höltje, H. & Karl, I. 2011. Assay conditions in laboratory experiments: is the use of constant rather than fluctuating temperatures justified when investigating temperature-induced plasticity? *Oecologia* **166**: 23–33.

Folguera, G., Bastías, D.A., Caers, J., Rojas, J.M., Piulachs, M.-D., Bellés, X., *et al.* 2011. An experimental test of the role of environmental temperature

variability on ectotherm molecular, physiological and life-history traits: implications for global warming. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **159**: 242–246.

Fox, C.W. 1993. Multiple mating, lifetime fecundity and female mortality of the bruchid beetle, Callosobruchus maculatus (Coleoptera: Bruchidae). *Funct. Ecol.* 203–208. JSTOR.

Fox, C.W., Bush, M.L., Roff, D.A. & Wallin, W.G. 2004a. Evolutionary genetics of lifespan and mortality rates in two populations of the seed beetle, Callosobruchus maculatus. *Heredity* **92**: 170–181.

Fox, C.W., Czesak, M.E. & Wallin, W.G. 2004b. Complex genetic architecture of population differences in adult lifespan of a beetle: Nonadditive inheritance, gender differences, body size and a large maternal effect. *J. Evol. Biol.* **17**: 1007–1017.

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., Ellison, S., *et al.* 2012. Package 'car.' *Vienna R Found. Stat. Comput.*

Fricke, C. & Arnqvist, G. 2004. Divergence in replicated phylogenies: the evolution of partial post-mating prezygotic isolation in bean weevils. *J. Evol. Biol.* **17**: 1345–1354. Wiley Online Library.

Gilchrist, G.W. 1995. Specialists and Generalists in Changing Environments. I. Fitness Landscapes of Thermal Sensitivity. *Am. Nat.* **146**: 252–270.

Hagstrum, D.W. & Leach, C.E. 1973. Role of Constant and Fluctuating Temperatures in Determining Development Time and Fecundity of Three Species of Stored-Products Coleoptera2. *Ann. Entomol. Soc. Am.* **66**: 407–410.

Hallsson, L.R. & Björklund, M. 2012a. Selection in a fluctuating environment and the evolution of sexual dimorphism in the seed beetle Callosobruchus maculatus. *J. Evol. Biol.* **25**: 1564–1575.

Hallsson, L.R. & Björklund, M. 2012b. Selection in a fluctuating environment leads to decreased genetic variation and facilitates the evolution of phenotypic plasticity: Evolutionary response in a fluctuating environment. *J. Evol. Biol.* **25**: 1275–1290.

Hansen, P.J. 2009. Effects of heat stress on mammalian reproduction. *Philos. Trans. R. Soc. B Biol. Sci.* **364**: 3341–3350.

Hartig, F. 2020. DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R package.

Hatfield, J.L. & Prueger, J.H. 2015. Temperature extremes: Effect on plant growth and development. *Weather Clim. Extrem.* **10**: 4–10.

Hedhly, A., Hormaza, J.I. & Herrero, M. 2009. Global warming and sexual plant reproduction. *Trends Plant Sci.* **14**: 30–36. Elsevier.

Hetem, R.S., Fuller, A., Maloney, S.K. & Mitchell, D. 2014. Responses of large mammals to climate change. *Temperature* **1**: 115–127.

Holt, R.D. 1990. The microevolutionary consequences of climate change. *Trends Ecol. Evol.* **5**: 311–315. Elsevier.

Huey, R.B., Berrigan, D., Gilchrist, G.W. & Herron, J.C. 1999. Testing the Adaptive Significance of Acclimation: A Strong Inference Approach. *Am. Zool.* **39**: 323–336.

Huey, R.B. & Hertz, P.E. 1984. Is a Jack-of-All-Temperatures a Master of None? *Evolution* **38**: 441–444.

Huey, R.B. & Kingsolver, J.G. 1989. Evolution of thermal sensitivity of ectotherm performance. *Trends Ecol. Evol.* **4**: 131–135.

Ivimey-Cook, E., Bricout, S., Candela, V., Maklakov, A.A. & Berg, E.C. 2021. Inbreeding reduces fitness of seed beetles under thermal stress. *J. Evol. Biol.* jeb.13899.

Janzen, D.H. 1967. Why Mountain Passes are Higher in the Tropics. *Am. Nat.* **101**: 233–249. [University of Chicago Press, American Society of Naturalists].

Johansson, F., Watts, P.C., Sniegula, S. & Berger, D. 2021. Natural selection mediated by seasonal time constraints increases the alignment between evolvability and developmental plasticity. *Evolution* **75**: 464–475.

Kassen, R. 2002. The experimental evolution of specialists, generalists, and the maintenance of diversity. *J. Evol. Biol.* **15**: 173–190.

Ketola, T., Mikonranta, L., Zhang, J., Saarinen, K., Örmälä, A.-M., Friman, V.-P., *et al.* 2013. FLUCTUATING TEMPERATURE LEADS TO EVOLUTION OF THERMAL GENERALISM AND PREADAPTATION TO NOVEL ENVIRONMENTS. *Evolution* **67**: 2936–2944. [Society for the Study of Evolution, Wiley].

Kingsolver, J.G. & Huey, R.B. 2008. Size, temperature, and fitness: three rules. 18.

Lashof, D.A. & Ahuja, D.R. 1990. Relative contributions of greenhouse gas emissions to global warming. *Nature* **344**: 529–531. Springer.

Lenth, R., Singmann, H., Love, J., Buerkner, P. & Herve, M. 2019. *emmeans: Estimated Marginal Means, aka Least-Squares Means (Version 1.3. 4)*.

Leslie, P.H. 1945. On the Use of Matrices in Certain Population Mathematics. *Biometrika* **33**: 183–212.

Lewontin, R.C. 1974. *The genetic basis of evolutionary change*. Columbia University Press New York.

Lopez, A.D. & Adair, T. 2019. Is the long-term decline in cardiovascular-disease mortality in high-income countries over? Evidence from national vital statistics net.

Magnusson, A., Skaug, H.J., Nielsen, A., Berg, C., Kristensen, K., Maechler, M., *et al.* 2019. Package 'glmmTMB. *R Package Version 01*.

Masson-Delmotte, V., Zhai, P. & Pirani, A. 2021. *IPCC, 2021: Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*.

McGuigan, K. & Sgro, C.M. 2009. Evolutionary consequences of cryptic genetic variation. *Trends Ecol. Evol.* **24**: 305–311. Elsevier.

Messina, F.J. 1991. Competitive interactions between larvae from divergent strains of the cowpea weevil (Coleoptera: Bruchidae). *Environ. Entomol.* **20**: 1438–1443. Oxford University Press Oxford, UK.

Messina, F.J. 2004. Predictable Modification of Body Size and Competitive Ability Following a Host Shift by a Seed Beetle. *Evolution* **58**: 2788–2797. [Society for the Study of Evolution, Wiley].

Mitchell, R. 1991. The traits of a biotype of Callosobruchus maculatus (F.)(Coleoptera: Bruchidae) from South India. *J. Stored Prod. Res.* **27**: 221–224. Elsevier.

Nevo, E. 1978. Genetic variation in natural populations: Patterns and theory. *Theor. Popul. Biol.* **13**: 121–177.

Niehaus, A.C., Angilletta, M.J., Sears, M.W., Franklin, C.E. & Wilson, R.S. 2012. Predicting the physiological performance of ectotherms in fluctuating thermal environments. *J. Exp. Biol.* **215**: 694–701.

Nylin, S. & Gotthard, K. 1998. Plasticity in Life-History Traits. *Annu. Rev. Entomol.* **43**: 63–83.

Paaby, A.B. & Rockman, M.V. 2014. Cryptic genetic variation: evolution's hidden substrate. *Nat. Rev. Genet.* **15**: 247–258.

Paaijmans, K.P., Heinig, R.L., Seliga, R.A., Blanford, J.I., Blanford, S., Murdock, C.C., *et al.* 2013. Temperature variation makes ectotherms more sensitive to climate change. *Glob. Change Biol.* **19**: 2373–2380.

Pachauri, R.K., Allen, M.R., Barros, V.R., Broome, J., Cramer, W., Christ, R., *et al.* 2014. *Climate change 2014: synthesis report. Contribution of Working Groups I, II and III to the fifth assessment report of the Intergovernmental Panel on Climate Change*. Ipcc.

Parmesan, C. 2006. Ecological and Evolutionary Responses to Recent Climate Change. *Annu. Rev. Ecol. Evol. Syst.* **37**: 637–669.

Pereira, H.M., Navarro, L.M. & Martins, I.S. 2012. Global Biodiversity Change: The Bad, the Good, and the Unknown. *Annu. Rev. Environ. Resour.* **37**: 25–50.

R Core Team. 2016. R: A language and environment for statistical computing. *R Found. Stat. Comput. Vienna Austria* **0**: {ISBN} 3-900051-07-0.

Rankin, D.J. & Arnqvist, G. 2008. Sexual Dimorphism Is Associated with Population Fitness in the Seed Beetle Callosobruchus Maculatus. *Evolution* **62**: 622–630.

Ratikainen, I.I. & Kokko, H. 2019. The coevolution of lifespan and reversible plasticity. *Nat. Commun.* **10**: 538.

Reboud, X. & Bell, G. 1997. Experimental evolution in Chiamydomonas. Ill. Evolution of specialist and generalist types in environments that vary in space and time. *Heredity* **78**: 507—514.

Rogell, B., Widegren, W., Hallsson, L.R., Berger, D., Björklund, M. & Maklakov, A.A. 2014. Sex-dependent evolution of life-history traits following adaptation to climate warming. *Funct. Ecol.* **28**: 469–478.

Stillwell, R.C. & Fox, C.W. 2007. Environmental effects on sexual size dimorphism of a seed-feeding beetle. *Oecologia* **153**: 273–280.

Stubben, C. & Milligan, B. 2007. Estimating and analyzing demographic models using the popbio package in R. *J. Stat. Softw.* **22**: 1–23.

Thompson, R.M., Beardall, J., Beringer, J., Grace, M. & Sardina, P. 2013. Means and extremes: building variability into community-level climate change experiments. *Ecol. Lett.* **16**: 799–806.

Trisos, C.H., Merow, C. & Pigot, A.L. 2020. The projected timing of abrupt ecological disruption from climate change. *Nature* **580**: 496–501. Nature Publishing Group.

Ursprung, C., Den Hollander, M. & Gwynne, D.T. 2009. Female seed beetles, Callosobruchus maculatus, remate for male-supplied water rather than ejaculate nutrition. *Behav. Ecol. Sociobiol.* **63**: 781–788. Springer.

Vasseur, D.A., DeLong, J.P., Gilbert, B., Greig, H.S., Harley, C.D.G., McCann, K.S., *et al.* 2014. Increased temperature variation poses a greater risk to species than climate warming. *Proc. R. Soc. B Biol. Sci.* **281**: 20132612.

Vasudeva, R., Deeming, D.C. & Eady, P.E. 2014. Developmental temperature affects the expression of ejaculatory traits and the outcome of sperm competition in Callosobruchus maculatus. *J. Evol. Biol.* **27**: 1811–1818.

Visser, M.E., Noordwijk, A.J. van, Tinbergen, J.M. & Lessells, C.M. 1998. Warmer springs lead to mistimed reproduction in great tits (Parus major). *Proc. R. Soc. Lond. B Biol. Sci.* **265**: 1867–1870. Royal Society.

Visser, M.E., te Marvelde, L. & Lof, M.E. 2012. Adaptive phenological mismatches of birds and their food in a warming world. *J. Ornithol.* **153**: 75–84.

Walters, R.J., Blanckenhorn, W.U. & Berger, D. 2012. Forecasting extinction risk of ectotherms under climate warming: an evolutionary perspective. *Funct. Ecol.* **26**: 1324–1338.

Wickham, H. 2009. *ggplot2 Elegant Graphics for Data Analysis*.