Evolution of phenotypic plasticity during environmental fluctuations

Running head: Evolution of phenotypic plasticity

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ZS, MIL, IIR and EB designed the experiment, ZS, ER and MT-B performed the phenotypic assays with the aid of MIL. EIFF and ZS analysed the data, with the aid of EB. ZS, MIL and EIFF drafted the manuscript. All authors contributed to the revision of the manuscript.

Acknowledgements

We thank Alexei Maklakov for access to the experimental evolution lines. This work was financed by the Swedish Research Council (Vetenskapsrådet, grant no. 2016-05195 and 2020-04388) and the Carl Tryggers Foundation (grant no. CTS 17:285) to MIL.

Conflict of Interest statement

The authors report no conflict of interest.

Data Availability Statement

Upon acceptance, the data will be archived at *Figshare*

Abstract

Evolution in fluctuating environments is predicted to disfavor specialization and instead select for alternative strategies, such as phenotypic plasticity or possibly bet-hedging, depending on the accuracy of environmental cues and type of fluctuations. While these two alternatives are often contrasted in theoretical studies, their evolution are seldom studied together in empirical work.

We used experimental evolution in the nematode worm *Caenorhabditis remanei* to simultaneously study the evolution of plasticity and bet-hedging in environments differing only in their temperature variability. We exposed worms for 30 generations to either fluctuating or slowly increasing temperature, these two environments had the same average temperature over evolutionary time. After experimental evolution, we scored size at sexual maturity and fitness in full siblings reared in two different temperatures, optimal 20°C and mildly stressful 25°C.

Experimental evolution in the fluctuating environment resulted in the evolution of increased body size plasticity but not increased bet-hedging, compared to evolution in the slowly changing environment. Plasticity followed the temperature size rule as size decreased with increasing temperature and this plastic response was adaptive. In addition, we documented substantial standing genetic variation in body size, which represents a potential for further evolutionary change.

Key words

Adaptation, Bet hedging, *Caenorhabditis remanei*, Experimental evolution, Phenotypic plasticity, Temperature

Introduction

Natural environments are generally not stable, but fluctuate on both long and short timescales, and a developing organism needs to take this environmental variation into account. While the parental environment can be a reliable cue if the environmental fluctuations are slow and predictable (Lachmann & Jablonka, 1996; Kuijper & Hoyle, 2015; Leimar & McNamara, 2015; Uller *et al.*, 2015) and can result in the evolution of anticipatory parental effects (Dey *et al.*, 2016; Lind *et al.*, 2020), faster and random fluctuations select against inheritance of the parental phenotype. Instead, phenotypic plasticity or bet-hedging is predicted to be adaptive (Moran, 1992; Simons, 2011). If the developmental environment provides reliable cues for the optimal offspring phenotype, theory predict the evolution of adaptive phenotypic plasticity (Moran, 1992; Gavrilets & Scheiner, 1993; Simons, 2011) which is the ability of a genotype to produce different phenotypes depending on environmental conditions (West-Eberhard, 2003). If plasticity is present, individuals will canalize fitness between environments by adjusting their phenotype according to the environmental conditions.

Alternatively, individuals may express bet-hedging, which acts to reduce variation in fitness (especially to avoid very low fitness values in certain environmental states) at the cost of lowered arithmetic mean fitness, often by producing offspring with a range of phenotypes (diversified bet-hedging), where some of the offspring always matches the environment and is successful (Philippi & Seger, 1989). In contrast to phenotypic plasticity, bet-hedging is generally seen as favored when environmental cues have low predictability (Cohen, 1966; Slatkin, 1974; Meyers & Bull, 2002; Kussell & Leibler, 2005; Wolf *et al.*, 2005), or when there is low correlation between the environment of development and selection (Tufto, 2015).

While both phenotypic plasticity and bet-hedging can be adaptive in variable environments (Simons, 2011; Furness *et al.*, 2015), most empirical studies of evolution in variable environments focus on the evolution of plasticity. While phenotypic plasticity is common and well documented (DeWitt & Scheiner, 2004), very few studies have investigated the evolution of plasticity. These studies generally follow two lines, either they investigate whether increased plasticity evolves in more variable environments (Moran, 1992; Tufto, 2015), or whether increased plasticity evolves as a (possibly transient) step in adaptation to a novel but stable environment (Lande, 2009; Chevin *et al.*, 2010; Levis & Pfennig, 2020). Studies focusing on the role of environmental heterogeneity have found stronger phenotypic plasticity in natural populations (Lind & Johansson, 2007; Lind *et al.*, 2011) or species (Hollander, 2008) originating from more variable environments. Moreover, two recent experimental evolution studies in microalgae have shown that variable environments can select for increased phenotypic plasticity (Schaum *et al.*, 2022), but that very unpredictable environments also can select against plasticity (Leung *et al.*, 2020). One possible explanation for selection against plasticity during rapid fluctuations is that it may take time for the plastic phenotype to be induced (Burton *et al.*, 2022; Dupont *et al.*, 2024). Studies that instead focus on the evolution of plasticity during adaptation to new stable environments have found that plasticity can rapidly evolve in a new environment (Sikkink *et al.*, 2014b; Corl *et al.*, 2018), but also that maladaptive plasticity can play a major role (Ghalambor *et al.*, 2015; Campbell-Staton *et al.*, 2021).

In contrast to phenotypic plasticity, empirical studies documenting bet-hedging are rare (Simons, 2011), and the few examples suggest that it is more likely to evolve if the environments differ dramatically in fitness (Bull, 1987), such as delayed germination in desert plants (Philippi, 1993; Clauss & Venable, 2000; Venable, 2007), diapause in killifish (Furness *et al.*, 2015) or experimental evolution in fluctuating environments with large fitness

differences (Beaumont *et al.*, 2009; Graham *et al.*, 2014). However, bet-hedging has been suggested as a mechanism explaining the strain-specific variance in reproduction between natural isolates of the nematode *Caenorhabditis elegans* (Diaz & Viney, 2014).

One environmental factor that is known to result in evolutionary adaptations (Berteaux *et al.*, 2004; Geerts *et al.*, 2015), but also alternative strategies, is temperature. Not only is temperature gradually increasing due to the ongoing climate change (Berteaux *et al.*, 2004), but climate change also results in increased temperature variability (Easterling *et al.*, 2000; Palmer & Räisänen, 2002; Van Aalst, 2006) potentially favoring evolution of increased plasticity or possibly bet-hedging. Indeed, most documented responses to climate change in natural populations are caused by pre-existing plasticity, while genetic adaptation seems rare (Merilä & Hendry, 2014).

Among traits showing plastic responses to temperature, body size is of immense importance to reproductive fitness. For females, a large body generally translates into increased egg production, and also males often benefit from large size (Hedrick & Temeles, 1989; Andersson, 1994). Therefore, perhaps surprisingly, in warm environments organisms generally increase growth rate, accelerate maturation but mature at a smaller size, which is called the temperature-size rule (Ray, 1960; Atkinson, 1994; Verberk *et al.*, 2021). This has sometimes been argued to be a passive by-product of other temperature-dependent processes (Atkinson, 1994; van der Have & de Jong, 1996; Forster *et al.*, 2011). However, small size may actually be actively regulated and beneficial in warm environments (Fryxell *et al.*, 2020; Verberk *et al.*, 2021) for example by being advantageous for thermoregulation (Partridge & Coyne, 1997), or allowing better regulation of oxygen demand and supply ratio (Walczyńska *et al.*, 2015; Verberk *et al.*, 2021).

We set out to investigate whether exposure to fluctuating temperature results in evolution of increased phenotypic plasticity compared to evolution in an environment with

147 slowly increased temperature and whether any plasticity in body size is adaptive, using
148 experimental evolution in the nematode *Caenorhabditis remanei*. *C. remanei* has a fast
149 generation time and, since it is dioecious, harbors substantial standing genetic variation
150 which makes it ideal for experimental evolution studies (Teotónio *et al.*, 2017). In addition,
151 *C. remanei* has been shown to respond to manipulations in temperature (Sikkink *et al.*,
152 2014a; Lind *et al.*, 2020), has the ability to respond plastically to new environmental
153 conditions (Lind *et al.*, 2020), and the plasticity to withstand heat-shock can itself evolve
154 (Sikkink *et al.*, 2014b). Body size of *C. remanei* is under directional selection towards an
155 optimum larger than the mean size under standard temperature conditions (Stångberg *et al.*,
156 2020), and pharmacologically lowered body size results in lower female reproduction (Lind
157 *et al.*, 2016). It's close relative *C. elegans* also follow the temperature-size rule (Kammenga
158 *et al.*, 2007). As an alternative to phenotypic plasticity, we also investigated if populations
159 evolving in variable environments had evolved increased diversifying bet-hedging.
160 We used previously established experimental evolution populations of *C. remanei*
161 (described in Lind *et al.*, 2020). During experimental evolution, replicate populations were
162 exposed to 30 generations in one of two regimes; *Fast temperature cycles* or *Increased*
163 *warming*. The *Fast temperature cycles* regime were switched between two temperatures
164 (20°C, 25°C) every second generation, and although the fluctuations are deterministic, it
165 represents an uncorrelated (and therefore unpredictable) fluctuating environment each
166 generation, as the next generation will either be in the same or in a different temperature. The
167 evolution in this environment was compared to the *Increased warming* regime, where worms
168 were exposed to experimental evolution in a gradually increasing temperature which slowly
169 raised from 20°C to 25°C over 30 generations, and which served as a control. Importantly,
170 these two regimes had the same average temperature (22.5°C) over evolutionary time, and
171 only differed in the rate and predictability of environmental change. While no theoretical

model to our knowledge have investigated evolution of plasticity in fluctuating versus slowly changing environments, it corresponds to different degrees of fluctuation, which is well explored theoretically (Moran, 1992; Gavrilets & Scheiner, 1993; Tufto, 2015). After the 30 generations of experimental evolution, we reared full-siblings in either standard 20°C, or warm 25°C, and scored them for reproduction and body size.

We predict that worms evolving in fluctuating temperature every 2nd generation would evolve increased phenotypic plasticity (relative to the *Increased warming* regime), since the timescale of this environmental variation is well within the parameter space where plasticity (and bet-hedging) is favored (Tufto, 2015). We predict that evolution of phenotypic plasticity is more likely than the evolution of bet hedging, since the differences in fitness between the two temperatures is likely to be relatively small (Lind *et al.*, 2020). If increased phenotypic plasticity has evolved, we predict that the *Fast temperature cycle* populations would show increased size difference between temperatures, but not increased phenotypic variance within one temperature. If instead increased diversifying bet-hedging had evolved, we predict that the *Fast temperature cycle* populations will show (1) increased within-family variance within each temperature, and (2) decreased heritability of size, because of an increased environmental component of phenotypic variance (Tufto, 2015). We also predict that any plasticity in body size will follow the temperature-size rule, and that this plasticity is adaptive.

194 **Methods**

195 *Experimental evolution*

196 For the experimental evolution we used *C. remanei* nematode worms, strain SP8 which has
197 been lab adapted for 15 generations at 20°C and subsequently exposed to 30 generations of
198 experimental evolution in two regimes (*Increased warming* and *Fast temperature cycles*).
199 The experimental evolution has been previously described in detail in Lind *et al.*, (2020b).
200 Briefly, in the *Increased warming* experimental evolution regime, the temperature gradually
201 raised from 20°C to 25°C, which is a novel and mildly stressful temperature. This gradual
202 change over 30 generations represent an increase of 0.1°C every 2.13 days and results in a
203 correlated parental and offspring environment. In the second regime, *Fast temperature*
204 *cycles*, the temperature fluctuated every second generation between 20°C and 25°C, resulting
205 in 14 temperature shifts but no exposure to the intermediate temperatures. The environmental
206 change is deterministic (every second generation) but since parents and offspring would end
207 up in either the same or in different temperature, it represents uncorrelated parental and
208 offspring environment. The generation time in *C. remanei* is temperature dependent; 4 days
209 long in 20°C and 3.4 days long in 25°C. Despite these differences, the average temperature
210 and the total chronological time of experimental evolution were identical for both regimes, at
211 22.5°C and 110 days respectively.

212 Each evolutionary regime consisted of six replicate populations. The populations were
213 maintained on 92 mm Petri dishes poured with NGM agar in climate chambers set to 60%
214 relative humidity. In order to prevent bacterial and fungal contamination, the agar and
215 bacterial LB contained the antibiotics streptomycin and kanamycin and the fungicide
216 nystatin. The plates were seeded with 2 ml of an antibiotic-resistant OP50-1 (pUC4K) strain
217 of *E. coli* (Stiernagle, 2006) that served as a source of food. Every 1-2 days, a piece of agar
218 containing approximately 150 worms of mixed ages was cut and transferred to a new plate

219 containing fresh bacteria. This resulted in populations with overlapping generations that were
220 maintained in a constant exponential growth phase. After the experimental evolution,
221 populations were expanded for two generations and frozen in -80°C for later revival and
222 subsequent phenotypic assays.
223

224 ***Experimental set-up***

225 Each replicate population of each of the two selection regimes was run in a separate block
226 resulting in 12 experimental blocks in total. For logistic reasons we focus on females, since
227 they are responsible for population growth rate and their fitness is straightforward to
228 measure.
229 Briefly, populations were revived from freezing and exposed to 25°C for 3
230 generations, to avoid any maternal effects associated with freezing. The third generation of
231 worms were split into eight families, each family consisting of one male and one female
232 worm. From each family, we randomly picked eight offspring females (full siblings) and
233 placed four females in 20°C and four in 25°C. Since our focus was evolution in females, their
234 fitness was assessed by mating them with standardized males from the ancestral line. Females
235 were kept together with two males (in case one of the males would be infertile/escape from
236 the plate). The ancestral line was, in contrast to selection regimes, maintained for three
237 generations in 20°C and subsequently split into 20°C and 25°C together with studied females.
238 For the detailed description of the experimental set up, see supplementary figure 1. In total,
239 we used 48 families per evolutionary regimes, and for each family we set up 4 offspring in
240 each temperature.
241

242 ***Phenotypic assays***

243 *Daily reproduction*

244 Female and male worms were transferred to a new plate every 24 hours. The old plate was
245 kept for two days to allow eggs to hatch and afterwards the number of viable offspring was
246 counted to determine age-specific reproduction and calculate individual fitness. In the case of
247 the males dying or escaping from the plate, they were replaced by a new male of the same
248 age from the ancestral line. The female worm was discarded after dying, or after three
249 consecutive days of zero reproduction.

250

251 *Body size*

252 The body size of the worm changes with time, reaching a peak before it declines (Lind *et al.*,
253 2016), and the age at maximum body size is temperature dependent. Worms in 20°C reach
254 their peak size at day 4 of adulthood (Lind *et al.*, 2016). The peak size in 25°C is on day 2 of
255 adulthood, which was determined during pilot assays. Photographs of worms were taken
256 during their peak size using a Lumenera Infinity2-5C digital microscope camera mounted on
257 a Leica M165C stereomicroscope. Body size was measured from the photographs using
258 *ImageJ 1.46r* (https://imagej.nih.gov/ij/) as total cross-section area.

259

260 ***Statistical analyses***

261 All statistical analyses were conducted in R 3.6.1 (R Core Team, 2015).

262

263 *Individual fitness*

264 We used the age-specific reproduction data to calculate rate-sensitive individual fitness λ_{ind}
265 for each individual (Brommer *et al.*, 2002), which is analogous to the intrinsic rate of
266 population growth (Stearns, 1992). Individual fitness was calculated by constructing a
267 population projection matrix for each individual, and then calculating the dominant

eigenvalue of this matrix, following McGraw & Caswell, (1996). Since we kept the population size and age structure constant during experimental evolution, individual fitness is maximized during evolution and is therefore the most appropriate fitness measure for this study (Mylius & Diekmann, 1995).

Thermal reaction norms

To test whether the degree of phenotypic plasticity has evolved, we used linear mixed-effect models to separately estimate the thermal reaction norms of body size and individual fitness, using the package *lme4* (Bates *et al.*, 2015) in R. The models included either body size (area) or individual fitness (λ_{ind}) as response variables. The full model included three fixed effects: mean-standardized temperature as a covariate, the experimental evolution regime as a categorical factor, and an interaction between temperature and evolution regime. We expect this interaction to be significant if the degree of plasticity has evolved during experimental evolution. Experimental line and dam identity were included as random effects. Significance of the fixed effects was evaluated using Wald χ^2 tests. Pseudo-R^2 values were calculated as the squared correlation coefficient between fitted values from the model and observed values.

Selection

To test if temperature responses in size are adaptive, we estimated the selection on body size and compared it to the observed temperature response. Selection on body size (area) was estimated using mixed-effect models in R with the package *lme4* and individual fitness (λ_{ind}) as the response variable. The full model included the following fixed effects: area, area^2, temperature (as a categorical factor with 2 levels), experimental evolution regime and all interactions between these variables except for interactions involving both area and area^2 together. Experimental line was included as random effect, assumed to only affect the

variance of the intercept. Significance of fixed effects was evaluated using Wald χ^2 tests. Pseudo-R^2 values were calculated as the squared correlation coefficient between fitted values from the model and observed values. The optimal size (i.e. the area that maximizes fitness) was calculated as: $-b/(2\times c)$, where b = the slope of the regression (i.e. the linear selection gradient) and c = the squared term of the regression (i.e. the quadratic selection gradient). Confidence intervals of the temperature-specific optimal sizes were generated by bootstrapping, implemented in the *boot* package using 10 000 bootstrap replicates.

Within family coefficient of variance

To test whether the degree of diversifying bet-hedging has evolved, we tested whether the experimental evolution regimes differed in the mean within family variance within temperatures. For each family, we used the trait values of the offspring (within a temperature) to calculate the within family variance. To account for differences in the means of the traits, we used within family means (i.e. the mean trait value of the family's offspring) to mean-standardize the variance by calculating the within family coefficient of variance (CV): $CV = \sigma/\mu$, where σ = within family standard deviation, and μ = within family mean. An ANOVA was used within each temperature to test if the evolution regimes differed in their mean within family CV.

Since it is more difficult to detect differences in variances than differences in means, we also performed power calculations on our ability to detect whether within-family CVs differ between the selective regimes. Balanced one-way ANOVA power calculations were performed to estimate the effect sizes possible to detect with power ranging from 0.70 to 0.95. Effect sizes, η^2, were obtained for our sample size of N = 48 per selection regime and a significance level of 0.05. η^2 is calculated as the sum of squared explained by the treatment

318 (here: selection regime) divided by the total sum of squares, and has an equivalent
319 interpretation as an R^2.
320
321 *Genetic variance and correlations*
322 For body size (cross-section area), genetic variance and genetic correlations across
323 temperature were estimated using animal models using the package *MCMCglmm* (Hadfield,
324 2010) in R. Univariate models were used to estimate genetic variance, whereas bivariate
325 models were used to estimate genetic correlations, both using Gaussian family for trait
326 distribution. An inverse Wishart prior with parameters V = 1 and nu = 0.02 were used in both
327 univariate and bivariate models. Pedigree data linking offspring to parents, based on full-sib
328 relationships, was included in the models. Convergence of the models were ensured by
329 evaluating diagnostic plots of posterior distributions, using the convergence diagnostic half-
330 width test by Heidelberger and Welch (1983), and by ensuring that the autocorrelation
331 between MCMC samples was close to zero.
332 For univariate models, body area was used as the response variable. Temperature (as a
333 categorical factor with 2 levels), experimental evolution regime, and an interaction between
334 the two, were included as fixed effects. Genetic variance (V_G), variance due to differences
335 between experimental lines, and residual variances were estimated separately as random
336 effects in the full model for each temperature-by-evolution regime combination. The full
337 model ran for 4.2×10^6 MCMC iterations, 0.2×10^6 samples were discarded as burnin, and the
338 thinning interval was 4000, resulting in a sample size of 1000 MCMC-samples. Reduced
339 models, subset by temperature-by-evolution regime combination, were used to assess the
340 statistical significance of V_G, by comparing the deviance information criterion (DIC) of
341 models with versus without genetic variance included.

342 Broad sense heritability ($H^2 = V_G/V_P$, where V_P = total phenotypic variance after
343 accounting for variance due to experimental line effects) and broad sense evolvability ($I^2 =$
344 $V_G/mean^2$, Hansen *et al.*, 2003, 2011) were used to estimate the population's evolutionary
345 potential of body size. This was estimated for each temperature-by-evolution regime
346 combination. Evolvability measures the expected percentage change in a trait per generation
347 under unit strength of selection. Compared to heritability, evolvability is independent from
348 the environmental variance and represents a measure of the evolutionary potential that is
349 comparable across traits, populations and species when applied to traits with a natural zero
350 and which are strictly positive (Hansen *et al.*, 2011).

351 Genetic correlations of body size were estimated using bivariate animal models in
352 *MCMCglmm.* The data was split in two subsets based on the experimental evolution regimes,
353 resulting in genetic correlations being estimated separately for the two evolution regimes.
354 Body area was the response variable and was treated as two traits in the models, as area at
355 20°C and area at 25°C. Random effects in the full models included genetic covariance
356 between the two temperatures, whereas V_G, variance due to differences between experimental
357 lines, and residual variances were estimated separately for each temperature. The full models
358 ran for 2.05×10^6 MCMC iterations, 0.05×10^6 samples were discarded as burnin, and the
359 thinning interval was 2000, resulting in a sample size of 1000 MCMC-samples. Reduced
360 models without genetic covariance were used to access the statistical significance of the
361 genetic covariance, by comparing the DIC of models with versus without genetic covariance
362 included. The genetic correlation of body size across temperatures was calculated by dividing
363 the genetic covariance by the product of the genetic standard deviation of the two
364 temperatures. This was done on the posterior distributions, in order to carry the error
365 forwards in the analyses.

366 To compare posterior distributions of H^2, I^2 and genetic correlations across
367 temperatures and selection regimes, we calculated, within each MCMC sample, the pairwise
368 differences in these measures and checked if the posterior distributions of these differences
369 had a 95% credibility interval that included zero. Pairwise comparisons of distributions were
370 only performed between evolution regimes within temperature, and between temperatures
371 within evolution regimes.
372
373
374 **Results**
375
376 Thermal reaction norms
377 *Size*
378 Size decreased significantly with increasing temperature (Wald $\chi^2 = 309.93$, df = 1, $p <$
379 0.001, Fig. 1A). There was a significant interaction between temperature and evolution
380 regime, where *Fast temperature cycles* had a steeper slope, meaning that it had evolved
381 increased plasticity in size (Wald $\chi^2 = 5.82$, df = 1, $p = 0.016$). However, the intercepts
382 (representing size at the mean temperature) were not significantly different between evolution
383 regime (Wald $\chi^2 = 0.09$, df = 1, $p = 0.769$). The models pseudo $R^2 = 0.51$. Variance
384 components: $V_{dam} = 25.03$, $V_{Line} = 20.92$, and $V_{residual} = 108.19$.
385
386 *Individual fitness (λ_{ind})*
387 The mean total reproduction decreased with temperature (mean ± SE: 20°C, 780 ± 17; 25°C,
388 672 ± 17; $p < 0.001$ for the difference between the temperatures). However, the individual
389 fitness (λ_{ind}) increased significantly with increasing temperature (Wald $\chi^2 = 3670$, df = 1, $p <$

0.001, Fig. 1B). The evolution regimes did not differ significantly in intercepts (Wald χ^2 = 0.02, df = 1, p = 0.887), nor was there a significant interaction between temperature and evolution regime (Wald χ^2 = 0.97, df = 1, p = 0.324). The best fit models pseudo R^2 = 0.86. Variance components: V_{dam} = 0.05, V_{Line} = 0.01, and $V_{residual}$ = 0.21.

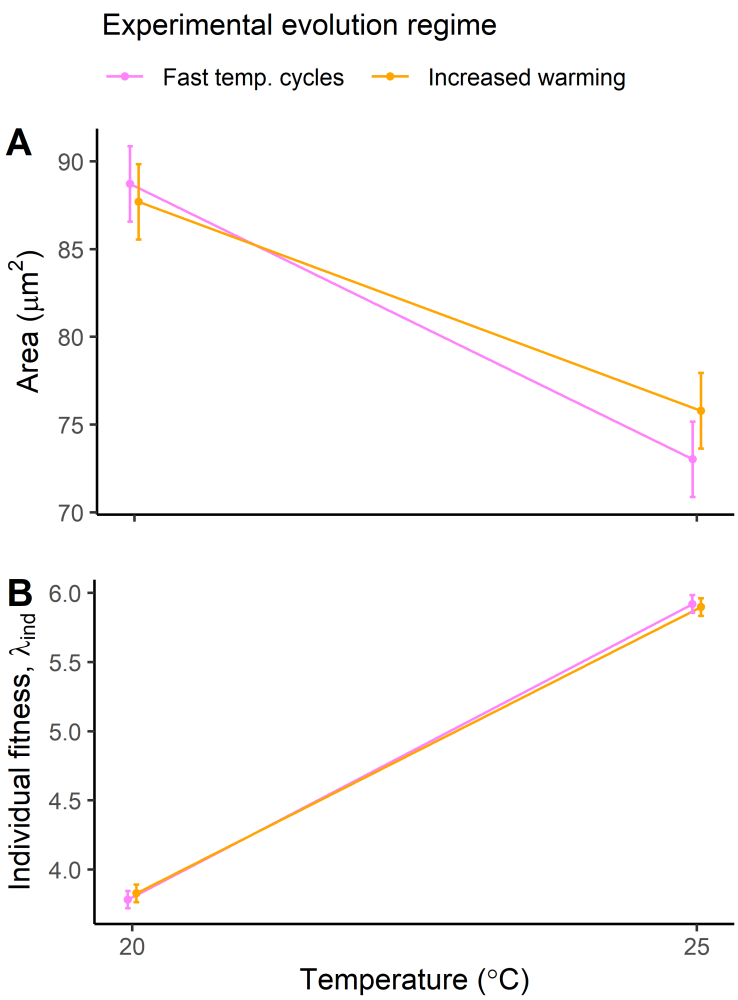

Figure 1. Thermal reaction norms showing means ± SE. (A) regression lines (with mean-standardized temperature): Fast; Area = 80.87 ± 2.08 – 3.14 ± 0.22 × Temperature. Increase; Area = 81.74 ± 2.08 – 2.38 ± 0.22 × Temperature. (B) regression lines (with mean-standardized temperature): Fast; λ_{ind} = 4.85 ± 0.06 + 0.43 ± 0.01 × Temperature. Increase; λ_{ind} = 4.86 ± 0.06 + 0.41 ± 0.01 × Temperature.

Selection

There was significant linear and quadratic selection on body size (linear slope: Wald χ^2 = 59.4, df = 1, p<0.001. Quadratic term: Wald χ^2 = 40.3, df = 1, p < 0.001). The selection differed significantly between temperatures (Fig. 2), given by a significant overall temperature effect (Wald χ^2 = 2992, df = 1, p < 0.001) and significant interaction effects between temperature and size (linear slope: Wald χ^2 = 13.3, df = 1, p < 0.001. Quadratic term: Wald χ^2 = 12.8, df = 1, p < 0.001). Maximum individual fitness (i.e. the optimal size) is predicted to be 93.73 µm at 20°C [95% bootstrap CI: 87.61, 112.98], and 84.19 µm at 25°C [95% bootstrap CI: 79.81, 92.61]. Selection was however not significantly different between the experimental evolution regimes (p > 0.22 for main effect and interactions between evolution regime and temperature or body size). No 3-way interaction between temperature, body size and evolution regime were significant (p > 0.18). The best fit model's pseudo R^2 = 0.85. Variance components: V_{Line} = 0.016, and $V_{residual}$ = 0.204.

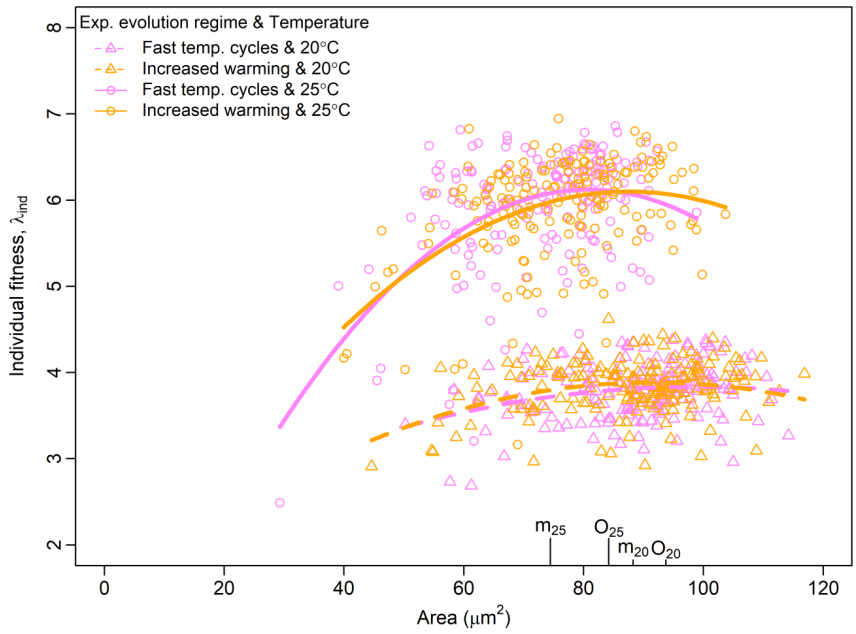

Figure 2. Selection on body size (area). Experimental evolution regimes were not statistically different, but are shown with separate lines. Overall regression line for 20°C: $\lambda_{ind} = 1.596 \pm 0.818 + 0.048 \pm 0.020 \times \text{Area} - 2.57\times10^{-4} \pm 1.16\times10^{-4} \times \text{Area}^2$. Overall regression line for 25°C: $\lambda_{ind} = 0.138 \pm 0.614 + 0.142 \pm 0.017 \times \text{Area} - 8.43\times10^{-4} \pm 1.20\times10^{-4} \times \text{Area}^2$. The mean size per temperature (m_{20}, and m_{25}) and optimal size (O_{20}, and O_{25}) are shown for 20°C and 25°C respectively. Individual fitness is higher in 25°C due to decreased development time, even if total reproduction is lower.

428 Within family CV

429 The evolution regimes did not differ significantly in within family CV of body size or of
430 individual fitness at either temperature (Table 1). Moreover, the distributions of within family
431 CV overlapped considerably between evolution regimes (Fig. 3). Power calculations showed
432 that we had a power of 90% to detect effects where the evolutionary regimes explained at
433 least 10% of the variation in within family CV (supplementary figure 2).

434

435

436

437 **Table 1.** Within family coefficient of variance (CV). Size (area) measured in μm^2, fitness as individual lambda
438 (λ_{ind})

439

Trait	**Temp. (°C)**	**Experimental evol. regime**	**Within family CV (mean±SE)**	**Difference between evolution regimes**	
				F (ndf=1, ddf=96)	**p**
Area	20	Fast temp. cycles	0.092 ± 0.009	3.799	0.054
		Increased warming	0.115 ± 0.009		
	25	Fast temp. cycles	0.100 ± 0.011	0.014	0.905
		Increased warming	0.102 ± 0.011		
Fitness	20	Fast temp. cycles	0.049 ± 0.005	0.266	0.608
		Increased warming	0.053 ± 0.005		
	25	Fast temp. cycles	0.087 ± 0.011	0.338	0.563
		Increased warming	0.079 ± 0.011		

440

441

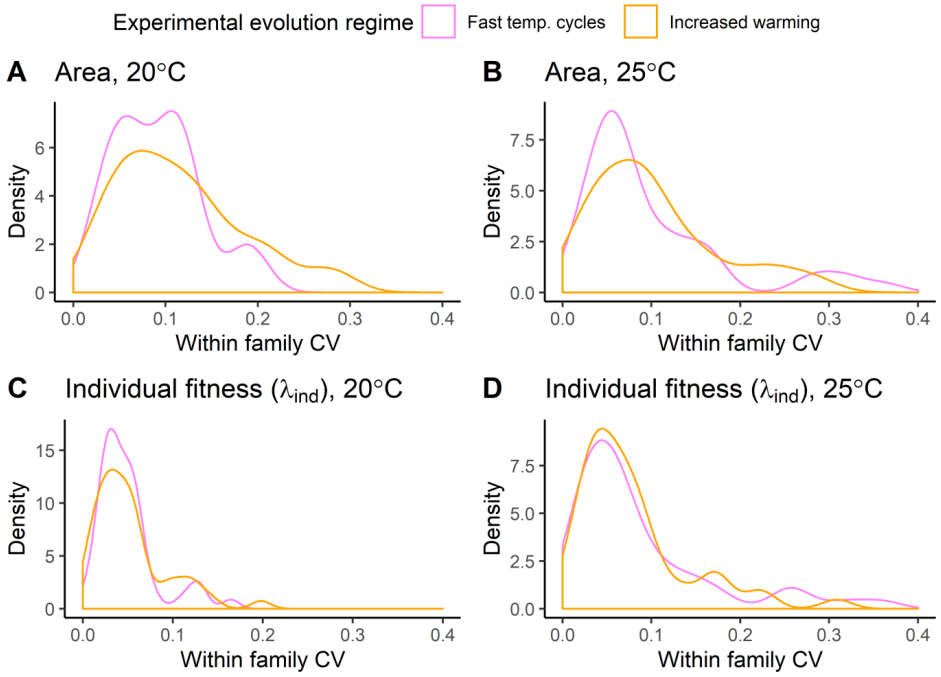

Figure 3. Distribution of within family coefficient of variance (CV) for two traits at two temperatures. The within family CV was estimated within temperature for each family by estimating the standard deviation (σ) and the mean (μ) of the family's offspring trait values, where CV = σ/μ. The density is the number of families.

<u>Genetic variance and correlations</u>

There was overall significant genetic variance for body size (measured as area) for the 4 combinations of temperature and evolution regime (models with genetic variance were at least 7 DIC lower compared to models without genetic variance, Table 2). There was also significant genetic covariance between temperatures for both evolution regimes (*fast temp. cycle*: model with covariance included was 2.04 DIC lower than model without covariance; *increased warming*: model with covariance was 2.79 DIC lower than model without covariance). However, the pairwise comparisons of the posterior distributions of heritability, evolvability and genetic correlations were not significantly different between the 4 different combinations of temperature and evolution regimes (all 95% credibility intervals of the pairwise differences contained zero).

Table 2. Heritability (H^2), I^2 (Evolvability, in percentage) and genetic correlations for body size (area).

Experimental evol. regime	Temp. (°C)	Posterior mode H^2 (95% CI)	Posterior mode I^2 (95% CI)	Posterior mode genetic correlation across temperatures (95% CI)
Fast temp. cycles	20	0.59 (0.44, 0.70)	1.13 (0.56, 2.01)	0.20 (-0.10, 0.38)
	25	0.56 (0.42, 0.66)	1.30 (0.53, 2.67)	
Increased warming	20	0.50 (0.37, 0.65)	1.08 (0.57, 2.42)	0.20 (-0.12, 0.41)
	25	0.49 (0.42, 0.67)	1.54 (0.69, 2.97)	

Discussion

We found that evolution in an environment that changed in temperature every 2nd generation (*Fast temperature cycles* regime) resulted in the evolution of increased phenotypic plasticity in body size. In contrast, we did not find any evidence of increased diversifying bet hedging in this evolutionary regime, since there was neither increased phenotypic variance within families nor reduced heritability.

Evolution in variable environments with no environmental correlations across generations is predicted to result in increased importance of either phenotypic plasticity or bet-hedging (Tufto, 2015). While phenotypic plasticity should be favored when the environment contains predictable cues for development, bet-hedging should be favored instead in less predictable environments (Botero *et al.*, 2015; Tufto, 2015). Moreover, the timescale of environmental variation relative to the generation time is also important, and when modeled by Tufto, (2015), environmental changes every 2nd generation is identified as

476 the intersection between the evolution of bet-hedging, reversible plasticity or developmental
477 plasticity. Since the *Fast temperature cycle* regime experienced fluctuations every 2nd
478 generation, they are ideally suited for investigating the evolution of plasticity and bet-hedging
479 in adult peak body size, an irreversible plastic trait closely connected to fitness.

480 We found evolution of increased phenotypic plasticity in the *Fast temperature cycle*
481 *regime*, manifested as a larger size difference between 20°C and 25°C (steeper reaction
482 norm). Evolution of increased phenotypic plasticity in more variable environments is
483 predicted by theory (Moran, 1992; Gavrilets & Scheiner, 1993; Kuijper & Hoyle, 2015), and
484 indeed studies using natural populations (Lind & Johansson, 2007; Lind *et al.*, 2011) or
485 species (Hollander, 2008) have found a positive correlation between the degree of
486 environmental heterogeneity and increased plasticity. Our study, using experimental
487 evolution, supports these results and pinpoint large temporal variation between temperature
488 extremes as the causative selection force underlying evolution of increased plasticity. This
489 has only been showed once before, in a recent experimental evolution study of the microalgae
490 *Thalassiosira pseudonana*, where populations evolving under fast fluctuations evolve
491 increased plasticity in photosynthesis (Schaum *et al.*, 2022). Our results also align with the
492 recent finding that laboratory-adapted populations of Zebra fish (*Danio rerio*), evolving in
493 very stable environments, have reduced physiological plasticity compared to their wild-
494 caught counterparts (Morgan *et al.*, 2022). Together, these studies demonstrate the
495 importance of environmental heterogeneity for the evolution of plasticity. It should however
496 be noted that very fast or unpredictable environmental change can make it impossible to
497 predict the environment, and in these circumstances plasticity may be selected against (Tufto,
498 2015), which was recently demonstrated using experimental evolution in the microalgae
499 *Dunaliella salina* (Leung *et al.*, 2020). Increased environmental variation is however not the
500 only factor that can influence the evolution of plasticity, but plasticity may also evolve when

501 a population is exposed to a novel (but stable) environment (Lande, 2009; Chevin *et al.*,
502 2010; Corl *et al.*, 2018). Evolution of increased plasticity has been shown for *C. remanei*
503 evolving in very heat-stressed environments (36.8°C), which demonstrates that evolution of
504 plasticity also can be a way to survive novel environment (Sikkink *et al.*, 2014b), a
505 phenomenon called plasticity-led evolution (Levis & Pfennig, 2020).
506
507 In contrast, we did not find any evolution of increased diversifying bet-hedging.
508 While empirical evidence for diversifying bet-hedging is much rarer than for phenotypic
509 plasticity, it is also much harder to detect since it is not trait means but trait variances that
510 needs to be measured. We have the power to detect differences in within-family CV where
511 the selection regimes explain at least 10% of the variation, which corresponds to a large
512 effect (Cohen, 1988). Still, there are a number of examples of bet-hedging, mainly regarding
513 delayed germination in desert plants (Philippi, 1993; Clauss & Venable, 2000; Venable,
514 2007), but also diapause in fish from ephemeral pools (Furness *et al.*, 2015). In addition,
515 diversifying bet-hedging has also evolved as a result of experimental evolution in
516 unpredictable environments in bacteria (Beaumont *et al.*, 2009) and fungi (Graham *et al.*,
517 2014). However, as predicted by Bull, (1987), a common factor in these examples is the very
518 strong fitness differences between environments, where one environmental state (for example
519 dry conditions) results in very low fitness. This contrasts to most examples of phenotypic
520 plasticity, where reproduction is possible to achieve in all environments, even if they are not
521 suitable without plastic adjustment of the phenotype. It has however been found that natural
522 isolates of *C. elegans* differ in their variance of total reproduction, which has been suggested
523 to result from a bet-hedging strategy where lines with more phenotypic variance may
524 originate from more variable environments (Diaz & Viney, 2014). We did not find any
525 difference in reproductive variance between our selection regimes, so further studies are

needed to understand why natural *C. elegans* isolates differ in their phenotypic variance of reproduction.

When exposed to increasing temperatures, organisms generally show phenotypic plasticity in size, and develop faster to mature smaller. Although there are exceptions, especially in terrestrial arthropods (Horne *et al.*, 2015), this relationship is so general that it is termed the temperature-size rule (Atkinson, 1994). Therefore, it is not surprising that plasticity in size was present in both evolutionary regimes, and like *C. elegans* (Kammenga *et al.*, 2007), we find that *C. remanei* follows the temperature-size rule. Importantly however, the regimes differed in the degree of size plasticity.

Whether the temperature-size rule (Atkinson, 1994) reflects an adaptive or non-adaptive response to temperature is not resolved. Arguments for it being non-adaptive center around constraints related to passive by-products of other temperature dependent processes (Atkinson, 1994; van der Have & de Jong, 1996; Forster *et al.*, 2011). For example, small body size can be a result of reduced growth rates which can be selected for as a by-product of increased reproductive investment in warmer temperatures (Heino & Kaitala, 1999; Walczyńska *et al.*, 2015), or, alternatively, as a trade-off between growth rate and resistance to oxidative stress (Kim *et al.*, 2011) which increases due to increased metabolism in higher temperatures (Birnie-Gauvin *et al.*, 2017).

However, small size in warm environments can also be adaptive. For example Fryxell *et al.*, (2020) showed that natural selection in warm temperatures favors smaller size in mosquitofish *Gambusia affinis* and a similar result has been reported in snails (Arendt, 2015). A possible advantage of small size in high temperatures can be a regulation of oxygen demand and supply ratio (Walczyńska *et al.*, 2015). In addition, as a body composed of small cells is more efficient in oxygen diffusion (Subczynski *et al.*, 1989; Verberk *et al.*, 2021) there will be a particularly strong selection pressure on organisms such as *Caenorhabditis*

551 nematodes, which have a fixed number of cells and thus the cell size determines the final
552 body size.

553 To assess whether temperature-induced plasticity in size is adaptive in *C. remanei*, we
554 compared individual fitness of different-sized individuals in both temperatures (Figure 2).
555 We found evidence of directional selection on increased size in both temperatures, but also
556 significant stabilizing selection within each temperature. Stabilizing selection implies that the
557 fitness optimum in both 20°C and 25°C was present in individuals within the data size-range
558 (as opposed to at extreme phenotypes). If small size in warm temperatures were maladaptive,
559 we would expect the largest individuals to have the highest fitness. In contrast, we found that
560 individuals both smaller and larger than the optimum size had decreased fitness. This
561 optimum size in the warm temperature was also substantially smaller than the optimum size
562 at the normal temperature, thus the plastic response to decrease size as a response to warm
563 temperature must be considered adaptive in *C. remanei*.

564 Interestingly, because most individuals raised in 25°C exhibit smaller size than would
565 be optimal (Figure 2.; mean size is smaller than optimal size), we can consider this
566 temperature plasticity to be a hyperplastic response, a special case of plasticity when plastic
567 response overshoots the optimum and brings individuals to the other side of the new fitness
568 peak (King & Hadfield, 2019). Since plasticity nevertheless increases fitness (compared to a
569 hypothetical non-plastic genotype), this hyperplasticity should still be considered adaptive. In
570 addition, we also found linear selection for large size in 20°C with individuals raised in 20°C
571 also having slightly smaller size than would be optimal. A possible explanation is a sexual
572 conflict between male and female worms, as males' optimal size is smaller than females'
573 optimal size in *C. remanei* (Stångberg *et al.*, 2020) so that males drag females from their
574 phenotypic optimum.

In contrast to size, we didn't find any difference in individual fitness between the regimes. While warm temperature caused a drop in total reproduction in both regimes, individuals raised in 25°C had significantly higher rate-sensitive individual fitness, which is a consequence of the temperature-induced alteration of the reproductive schedule, including a faster development time (Sekajova *et al.*, 2022).

Previous experimental evolution and artificial selection studies in the SP8 line of *C. remanei*, which was our founder population, have documented fast evolutionary responses to selection in life history traits, suggesting a large amount of standing genetic variation (Chen & Maklakov, 2012; Zwoinska *et al.*, 2016; Lind *et al.*, 2020). Accordingly, we found substantial genetic variation for size, for all treatment × temperature combinations, which not only allowed the lines to respond to selection, but also represents a potential for further evolution. Since we used full-sibs, our estimates of genetic variance could potentially be inflated by the presence of dominance variance and epistatic interactions. Epistatic interactions are present for body size in the sister species *C. elegans* (Noble *et al.*, 2017; Maulana *et al.*, 2022), but while most genetic variance for this trait was found to be additive and with similar additive heritability to our estimate using an experimental evolution design (Noble *et al.*, 2017), assessments of narrow-sense heritability using recombinant inbreed lines found that additive effects played a smaller role for body-size (Maulana *et al.*, 2022). Therefore, we assume that our broad sense heritability overestimates the additive genetic effect, but to an unknown degree. Moreover, we didn't observe any significant differences in broad-sense heritability between the lines, which further support our evidence of no evolution of diversified bet-hedging, which comes with the prediction of lowered heritability in traits (Tufto, 2015), nor did we observe any genetic correlations between trait values in the two temperatures, suggesting that traits can largely evolve independently in each environment.

Conclusion

To summarize, we found that 30 generations of experimental evolution in a heterogeneous environment (*Fast temperature cycles)* resulted in the evolution of increased phenotypic plasticity, compared to evolution in a slowly changing environment (*Increased warming)*. We showed that plasticity followed the temperature size rule and was adaptive. In addition, substantial amount of standing genetic variation found in the line represents a potential for further evolutionary change.

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