Evolution during uncorrelated environmental fluctuations: bet-hedging or phenotypic plasticity?

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Abstract

Evolution in fluctuating environments is predicted to select for phenotypic plasticity or 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 fast 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 discovered substantial standing genetic variation in body size, which represents a potential for further evolutionary change.

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), fast 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. In a temporally variable environment, bet-hedging 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 (Philippi & Seger, 1989). This can be achieved in two ways; either by producing offspring with an average generalist phenotype that does reasonably well in all environments (conservative bet-hedging), or 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). Both bet-hedging strategies reduce short term (arithmetic mean) fitness by maximizing long term (geometric mean) fitness (Slatkin, 1974). 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 have however 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).

In contrast to phenotypic plasticity, empirical studies documenting bet-hedging are rare (Simons, 2011), mostly focused on diversifying bet-hedging and only few studies have been able to document its presence. The most well-known example of bet-hedging is delayed germination of desert plants, where variance in fitness between years explains variation in germination (Simons, 2009) and populations or species with more environmental variation between years have a greater degree of diversifying bet-hedging in germination time (Philippi, 1993; Clauss & Venable, 2000; Venable, 2007). In addition, two experimental evolution studies have documented the evolution of diversifying bet-hedging in unpredictable environments in bacteria (Beaumont *et al.*, 2009) and fungus (Graham *et al.*, 2014). Besides those examples, there is a lack of studies on bet-hedging, especially in animals (but see Furness *et al.*, (2015) on wild fish populations).

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 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 males also 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). 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) for example by being advantageous for thermoregulation (Partridge & Coyne, 1997), or allowing better regulation of oxygen demand and supply ratio (Walczyńska *et al.*, 2015).

We set out to investigate whether exposure to fluctuating temperature results in evolution of increased phenotypic plasticity or bet-hedging, and whether any plasticity in body size is adaptive, using experimental evolution in the nematode *Caenorhabditis remanei*. *C. remanei* has a fast generation time and, since it is dioecious, harbors substantial standing genetic variation which makes it ideal for experimental evolution studies (Teotónio *et al.*, 2017). In addition, *C. remanei* has been shown to respond to manipulations in temperature (Sikkink *et al.*, 2014; Lind *et al.*, 2020) and has the ability to respond plastically to new environmental conditions (Lind *et al.*, 2020). Body size of *C. remanei* is under positive yet balancing selection under standard temperature conditions (Stångberg *et al.*, 2020), and pharmacologically lowered body size results in lower female reproduction (Lind *et al.*, 2016).

We used previously established experimental evolution populations of *C. remanei* (described in Lind *et al.*, (2020). During experimental evolution, replicate populations were exposed to 30 generations in one of two regimes; *Fast temperature cycles* or *Increased warming*. The *Fast temperature cycles* regime represents an uncorrelated (and therefore unpredictable) fluctuating environment between generations. The evolution in this environment was compared to the *Increased warming* regime, where worms were exposed to experimental evolution in a gradually increasing temperature which slowly raised from 20°C to 25°C over 30 generations, and which served as a control. Importantly, these two regimes had the same average temperature (22.5°C) over evolutionary time, and only differed in the rate and predictability of environmental change. 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 either increased phenotypic plasticity or bet-hedging (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). 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 instead predict that the *Fast temperature cycle* populations would 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 would follow the temperature-size rule, and that this plasticity is adaptive.

Methods

Experimental evolution

For the experimental evolution we used *C. remanei* nematode worms, strain SP8 which has been lab adapted for 15 generations at 20°C and subsequently exposed to 30 generations of experimental evolution in two regimes (*Increased warming* and *Fast temperature cycles*). The experimental evolution has been previously described in detail in Lind *et al.*, (2020b). Briefly, in the *Increased warming* experimental evolution regime, the temperature gradually raised from 20°C to 25°C, which is a novel and mildly stressful temperature. This gradual change over 30 generations represent an increase of 0.1°C every 2.13 days and results in a correlated parental and offspring environment. In the second regime, *Fast temperature cycles*, the temperature fluctuated every second generation between 20°C and 25°C, resulting in 14 temperature shifts and uncorrelated parental and offspring environment. The generation time in *C. remanei* is temperature dependent; 4 days long in 20°C and 3.4 days long in 25°C. Despite these differences, the average temperature and the total chronological time of experimental evolution were identical for both regimes, at 22.5°C and 110 days respectively.

Each evolutionary regime consisted of six replicate populations. The populations were maintained on 92 mm Petri dishes poured with NGM agar in climate chambers set to 60% relative humidity. In order to prevent bacterial and fungal contamination, the agar and bacterial LB contained the antibiotics streptomycin and kanamycin and the fungicide nystatin. The plates were seeded with 2 ml of an antibiotic-resistant OP50-1 (pUC4K) strain of *E. coli* (Stiernagle, 2006) that served as a source of food. Every 1-2 days, a piece of agar containing approximately 150 worms of mixed ages was cut and transferred to a new plate containing fresh bacteria. This resulted in populations with overlapping generations that were maintained in a constant exponential growth phase. After the experimental evolution, populations were expanded for two generations and frozen in -80°C for later revival and subsequent phenotypic assays.

Experimental set-up

Each replicate population of each of the two selection regimes was run in a separate block resulting in 12 experimental blocks in total. For logistic reasons we focus on females, since they are responsible for population growth rate and their fitness is straightforward to measure.

Briefly, populations were revived from freezing and exposed to 25°C for 3 generations, to avoid any maternal effects associated with freezing. The third generation of worms were split into eight families, each family consisting of one male and one female worm. From each family, we randomly picked eight offspring females (full siblings) and placed four females in 20°C and four in 25°C. Since our focus was evolution in females, their fitness was assessed by mating them with standardized males from the ancestral line. Females were kept together with two males (in case one of the males would be infertile/escape from the plate). The ancestral line was, in contrast to selection regimes, maintained for three generations in 20°C and subsequently split into 20°C and 25°C together with studied females. For the detailed description of the experimental set up, see supplementary figure 1.

Phenotypic assays

Daily reproduction

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

Body size

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

Statistical analyses

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

Individual fitness

We used the age-specific reproduction data to calculate rate-sensitive individual fitness λ_{ind} for each individual (Brommer *et al.*, 2002), which is analogous to the intrinsic rate of population growth (Stearns, 1992). Individual fitness was calculated by constructing a 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, assumed to only affect the variance of the intercepts. 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. A mean CV was calculated for each temperature-by-evolution regime combination (*fast temp. cycles* in 20°C; *fast temp. cycles* in 25°C; *increased warming* in 20°C; *increased warming* in 25°C). The mean CVs were then compared across temperatures using ANOVA. As this test does not account for the uncertainty while estimating the means, it will be non-conservative and more likely to give false-positive results (type-I errors).

Genetic variance and correlations

For body size (area), genetic variance and genetic correlations across temperature were estimated using animal models in the package *MCMCglmm* (Hadfield, 2010) in R. Univariate models were used to estimate genetic variance, whereas bivariate models were used to estimate genetic correlations, both using Gaussian family for trait distribution. An inverse Wishart prior with parameters V = 1 and nu = 0.02 were used in both univariate and bivariate models. Pedigree data linking offspring to parents, based on full-sib relationships, was included in the models. Convergence of the models were ensured by evaluating diagnostic plots of posterior distributions, using the convergence diagnostic half-width test by Heidelberger and Welch (1983), and by ensuring that the autocorrelation between MCMC samples was close to zero.

For univariate models, body area was used as the response variable. Temperature (as a categorical factor with 2 levels), experimental evolution regime, and an interaction between the two, were included as fixed effects. Genetic variance (V_G), variance due to differences between experimental lines, and residual variances were estimated separately as random effects in the full model for each temperature-by-evolution regime combination. The full model ran for 4.2×10^6 MCMC iterations, 0.2×10^6 samples were discarded as burnin, and the thinning interval was 4000, resulting in a sample size of 1000 MCMC-samples. Reduced models, subset by temperature-by-evolution regime combination, were used to assess the statistical significance of V_G, by comparing the deviance information criterion (DIC) of models with versus without genetic variance included.

Broad sense heritability ($H^2 = V_G/V_P$, where V_P = total phenotypic variance after accounting for variance due to experimental line effects) and broad sense evolvability (I^2 =

V_G/mean^2, Hansen *et al.*, 2003, 2011) were used to estimate the population's evolutionary potential of body size. This was estimated for each temperature-by-evolution regime combination. Evolvability measures the expected percentage change in a trait per generation under unit strength of selection. Compared to heritability, evolvability is independent from the environmental variance and represents a measure of the evolutionary potential that is comparable across traits, populations and species (Hansen *et al.*, 2011).

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

To compare posterior distributions of H^2, I^2 and genetic correlations, we calculated a 95% resampling interval for the p-value that tests if the distributions are different from each other. Each p-value was calculated as p = 1 - mean (distribution$_1$ - distribution$_2$ > 0), where the posterior mode of distribution$_1$ is larger than the posterior mode of distribution$_2$. To ensure that the order of the samples within the distributions is independent, we did 1000 resamples without replacement of each distribution, and calculated a p-value for each of these resampling events. This allowed us to estimate a 95% resampling interval of p-values for each comparison of posterior distributions. Pairwise comparisons of distributions were only performed between evolution regimes within temperature, and between temperatures within evolution regimes.

Results

Thermal reaction norms

Size

Size decreased significantly with increasing temperature (Wald $\chi^2 = 309.93$, df = 1, p < 0.001, Fig. 1A). There was a significant interaction between temperature and evolution regime, where *Fast temperature cycles* had a steeper slope, meaning that it had evolved increased plasticity in size (Wald $\chi^2 = 5.82$, df = 1, p = 0.016). However, the intercepts (representing size at the mean temperature) were not significantly different between evolution regime (Wald $\chi^2 = 0.09$, df = 1, p = 0.769). The models pseudo $R^2 = 0.51$. Variance components: $V_{dam} = 25.03$, $V_{Line} = 20.92$, and $V_{residual} = 108.19$.

Individual fitness (λ_{ind})

The mean total reproduction decreased with temperature (mean ± SE: 20°C, 780 ± 17; 25°C, 672 ± 17; $p < 0.001$ for the difference between the temperatures). However, the individual 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 $\chi^2 = 0.02$, df = 1, $p = 0.887$), nor was there a significant interaction between temperature and evolution regime (Wald $\chi^2 = 0.97$, 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 $V_{residual} = 0.21$.

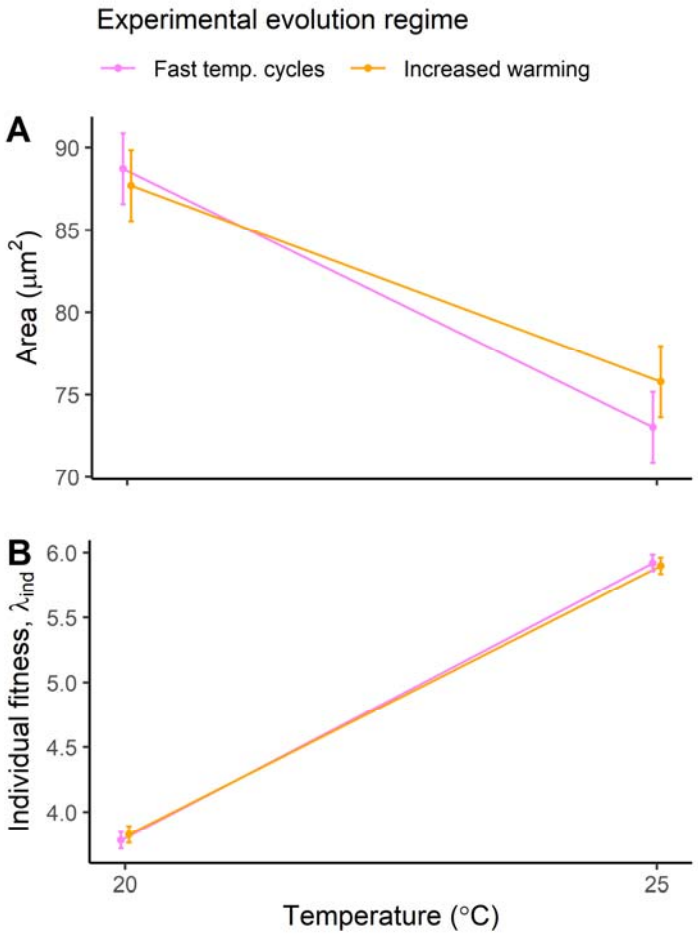

Figure 1. Thermal reaction norms showing means ± SE. (A) regression lines (with mean-standardized temperature): Fast; Area = 80.87 ± 2.08 – 3.14 ± 0.22 × Temperature. Increase; Area = 81.74 ± 2.08 – 2.38 ± 0.22 × Temperature. (B) regression lines (with mean-standardized temperature): Fast; λ_{ind} = 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 $\chi^2 = 59.4$, df = 1, $p<0.001$. Quadratic term: Wald $\chi^2 = 40.3$, df = 1, $p < 0.001$). The selection differed significantly between temperatures (Fig. 2), given by a significant overall temperature effect (Wald $\chi^2 = 2992$, df = 1, $p < 0.001$) and significant interaction effects between temperature and size (linear slope: Wald $\chi^2 = 13.3$, 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 was significant (p > 0.18). The best fit models pseudo R^2 = 0.85. Variance components: V_{Line} = 0.016, and $V_{residual}$ = 0.204.

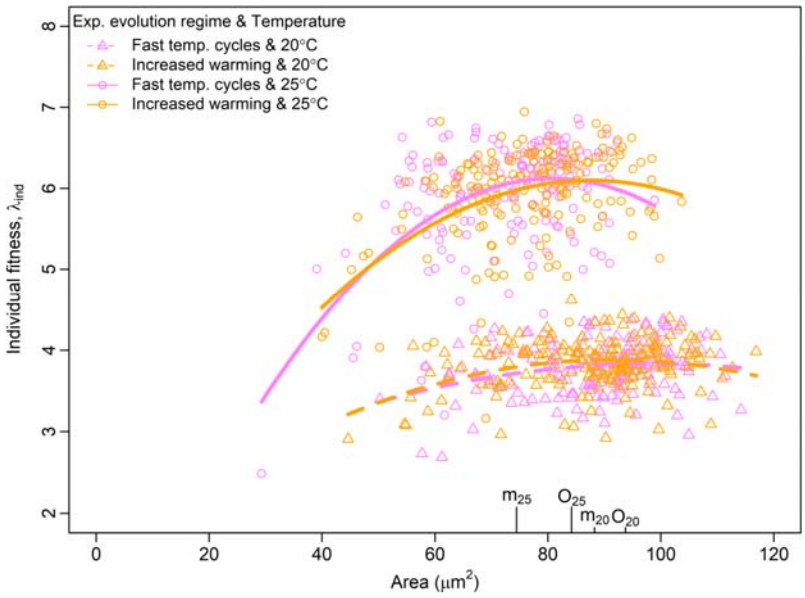

Figure 2. Selection on body size (area). Experimental evolution regimes were not statistically different, but are shown with separate lines. Overall regression line for 20°C: λ_{ind} = 1.596 ± 0.818 + 0.048 ± 0.020 × Area – $2.57\times10^{-4} \pm 1.16\times10^{-4}$ × Area^2. Overall regression line for 25°C: λ_{ind} = 0.138 ± 0.614 + 0.142 ± 0.017 × Area – $8.43\times10^{-4} \pm 1.20\times10^{-4}$ × 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.

Within family CV

The evolution regimes did not differ significantly in within family CV of body size or of individual fitness at either temperature (Table 1). Moreover, the distributions of within family CV overlapped considerably between evolution regimes (Fig. 3).

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

Trait	Temp. (°C)	Experimental evol. regime	Within family CV (mean±SE)	Difference between evolution regimes	
				F (df=1)	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		

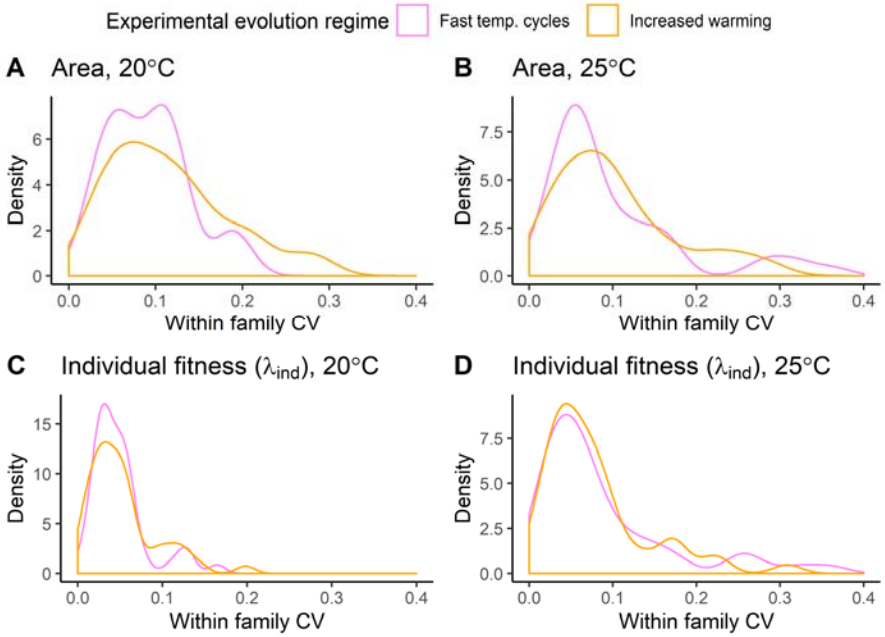

Figure 3. Distribution of within family coefficient of variance (CV) for two traits at two temperatures. The within family CV was estimated within temperature for each family by estimating the standard deviation (σ) and the mean (μ) of the family's offspring trait values, where CV = σ/μ. The density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

Genetic variance and correlations

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 p-values within the 95% resampling interval > 0.20).

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

We found evolution of increased phenotypic plasticity in the *Fast temperature cycle regime*, manifested as a larger size difference between 20°C and 25°C (steeper reaction norm). Evolution of increased phenotypic plasticity in more variable environments is predicted by theory (Moran, 1992; Gavrilets & Scheiner, 1993; Kuijper & Hoyle, 2015), and indeed studies using natural populations (Lind & Johansson, 2007; Lind *et al.*, 2011) or species (Hollander, 2008) have found a positive correlation between the degree of environmental heterogeneity and increased plasticity. Our study, using experimental evolution, support these results and pinpoint environmental heterogeneity as the causative

selection force underlying evolution of increased plasticity. This has only been showed once before, in a recent experimental evolution study of the microalgae *Thalassiosira pseudonana*, where populations evolving under fast fluctuations evolve increased plasticity in photosynthesis (Schaum *et al.*, 2022). Our results also align with the recent finding that laboratory-adapted populations of Zebra fish (*Danio rerio*), evolving in very stable environments, have reduced physiological plasticity compared to their wild-caught counterparts (Morgan *et al.*, 2022). Together, these studies demonstrate the importance of environmental heterogeneity for the evolution of plasticity. It should however be noted that very fast or unpredictable environmental change can make it impossible to predict the environment, and in these circumstances plasticity may be selected against (Tufto, 2015), which was recently demonstrated using experimental evolution in the microalgae *Dunaliella salina* (Leung *et al.*, 2020).

In contrast, we did not find any evolution of increased diversifying bet-hedging. While empirical evidence for diversifying bet-hedging is much rarer than for phenotypic plasticity, it is also much harder to detect since it is not trait means but trait variances that needs to be measured. Still, there are a number of examples of bet-hedging, mainly regarding delayed germination in desert plants (Philippi, 1993; Clauss & Venable, 2000; Venable, 2007), but also diapause in fish from ephemeral pools (Furness *et al.*, 2015). In addition, diversifying bet-hedging has also evolved as a result of experimental evolution in unpredictable environments in bacteria (Beaumont *et al.*, 2009) and fungi (Graham *et al.*, 2014). However, as predicted by Bull, (1987), a common factor in these examples is the very strong fitness differences between environments, where one environmental state (for example dry conditions) results in very low fitness. This contrasts to most examples of phenotypic plasticity, where reproduction is possible to achieve in all environments, even if they are not suitable without plastic adjustment of the phenotype.

While phenotypic plasticity is often contrasted to diversifying bet-hedging, much less focus has been on conservative bet-hedging. Conservative bet-hedging also reduces short-term arithmetic mean fitness in order to maximize long-term geometric mean fitness, but unlike diversifying bet-hedging it does not produce a range of phenotypes (Olofsson *et al.*, 2009). Traditionally, conservative bet-hedging has thus been interpreted as a production of generalist phenotypes, suited for an average environment (Philippi & Seger, 1989). However, phenotypic plasticity should, in some cases, also be considered a type of conservative bet-hedging (Haaland *et al.*, 2021). This occurs when plasticity results in lower variance in fitness (due to the canalization of fitness) and, at the same time, lower expected arithmetic fitness but higher geometric mean fitness. Lowered arithmetic fitness could result from costs of plasticity, which reduces maximal expected fitness (Haaland *et al.*, 2021). Therefore, to determine whether plasticity is a type of conservative bet-hedging, one needs to have information on all the three factors (variance in fitness, arithmetic and geometric mean fitness). Thus, it might be difficult to distinguish between them empirically.

Since the two experimental evolution regimes differ in the degree of plasticity in body size, but not in fitness, our results suggest that the increased plasticity in the *Fast cycle regime* is not resulting in lowered arithmetic fitness, thus that plasticity is not mainly a conservative bet-hedging strategy in this case. However, more research across many traits and organisms is needed to investigate whether phenotypic plasticity generally can be considered a conservative bet-hedging strategy.

When exposed to increasing temperatures, organisms generally show phenotypic plasticity in size, and develop faster to mature smaller. Although there are exceptions (Rogell *et al.*, 2014), 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. 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) there will be a particularly strong selection pressure on organisms such as *Caenorhabditis* nematodes, which have a fixed number of cells and thus the cell size determines the final body size.

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

Interestingly, because most individuals raised in 25°C exhibit smaller size than would be optimal (Figure 2.; mean size is smaller than optimal size), we can consider this temperature plasticity to be a hyperplastic response, a special case of plasticity when plastic response overshoots the optimum and brings individuals to the other side of the new fitness peak (King & Hadfield, 2019). Since plasticity nevertheless increases fitness (compared to a hypothetical non-plastic genotype), this hyperplasticity should still be considered adaptive. In addition, we also found linear selection for large size in 20°C with individuals raised in 20°C also having slightly smaller size than would be optimal. A possible explanation is a sexual conflict between male and female worms, as males' optimal size is smaller than females' optimal size in *C. remanei* (Stångberg *et al.*, 2020) so that males drag females from their 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. However, while epistatic interactions are present for body size in the sister species *C. elegans*, most genetic variance for this trait is additive and with similar additive heritability to our estimate (Noble *et al.*, 2017). 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.

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 but not bet-hedging, 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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Author contributions

ZS, MIL, IIR and EB designed the experiment, ZS, ER and MTB 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.

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