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Experimental evolution in fluctuating environments: tolerance measurements at constant temperatures incorrectly predict the ability to tolerate fluctuating temperatures

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Abstract

The ability to predict the consequences of fluctuating environments on species distribution and extinction often relies on determining the tolerances of species or genotypes in different constant environments (i.e. determining tolerance curves). However, very little is known about the suitability of measurements made in constant environments to predict the level of adaptation to rapidly fluctuating environments. To explore this question, we used bacterial clones adapted to constant or fluctuating temperatures and found that measurements across a range of constant temperatures did not indicate any adaptation to fluctuating temperatures. However, adaptation to fluctuating temperatures was only apparent if growth was measured during thermal fluctuation. Thus, tolerance curves based on measurements in constant environments can be misleading in predicting the ability to tolerate fast environmental fluctuations. Such complications could lead to false estimates of the genetic merits of genotypes and extinction risks of species due to climate change-induced thermal fluctuations.

Introduction

The most typical way to describe the level of adaptation of a species or a genotype to its environment and to environmental variation is by measuring tolerance, or performance, across a range of constant environments (i.e. determining the tolerance curve of a species) (Huey & Kingsolver, 1993). Poor performance in certain environments, such as at hot or cold temperatures, suggests a limited capability to tolerate these conditions. However, overall low elevation of thermal tolerance curve suggests a generally poor performance regardless of the temperature. Indeed, theories predict that tolerance to extreme thermal environments should evolve in fluctuating thermal environments, especially if the fluctuations occur within generations (Levins, 1968; Lynch & Gabriel, 1987). Alternatively, fluctuations could select for the higher elevation of the tolerance curve, that is overall improved performance (Scheiner & Yampolsky, 1998; Ketola *et al.*, 2013, 2014). For example, if the ability to change the shape of the tolerance curve is limited, the only possibility to improve performance at extremes is via increased tolerance curve elevation. The shape and elevation of tolerance curves are also used in ranking genotypes and species for their suitability for tolerating variable environments (Dobzhansky & Spassky, 1963; Falconer & Mackay, 1996). Moreover, they are used for predicting extinction risks and the invasion ability of species and populations (Lee & Gelembiuk, 2008).

However, modifications in tolerance curves are not the only evolutionary solutions for coping with environmental heterogeneity. Rapidly fluctuating environments could also select for an improved ability to withstand environmental fluctuations by other means, for example via inducible phenotypic plasticity (Levins, 1968; DeWitt & Langerhans, 2004), such as the expression of heat-shock proteins during short-term stress (Sorensen *et al.*, 2003; Ketola *et al.*, 2004). However, such adaptations to fluctuating environments might not be visible in tolerance curves measured in constant environments (Schulte *et al.*, 2011; Ketola *et al.*, 2014; Magalhães *et al.*, 2014).

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To test whether fluctuating environments select for changes in tolerance curves (measured in constant environments), we experimentally manipulated *Serratia marcescens* bacteria with thermal fluctuations over evolutionary timescales. In this experiment, we reared replicated ($n = 10$) *S. marcescens* populations from the same clonal origin for 2 months at a constant temperature of 31 °C or at two different rapidly fluctuating (ca. 1 h interval) temperature regimes with a mean temperature of 31 °C. In the first fluctuating regime, the fluctuations occurred more smoothly as the changes between stressful temperatures (24 and 38 °C) were preceded by an intermediate step at a more optimal temperature (31 °C), hereafter named smooth fluctuations. In the second fluctuating environment treatment, bacteria experienced abrupt changes between the stressful temperatures (24 and 38 °C), hereafter named abrupt fluctuations. These two fluctuating treatments were originally designed to explore hypotheses that are not concerned in this manuscript, and we do not have *a priori* hypotheses for thermal tolerance differences between these two treatments. Here, we concentrate on comparing differences between constant and fluctuating treatments. The fluctuations used are more rapid than the bacteria are likely to experience in the wild. This test, however, was designed to test theories of thermal adaptation that underline the strong role of fast 'within generation' variation (Levins, 1968; Lynch & Gabriel, 1987). After 2 months of evolution in their respective environments, a total of 360 clones (12 clones from each replicate population) were measured for their maximal growth rate and biomass yield at six constant temperatures. Both growth rate and yield can be advantageous to the bacteria in our experimental settings; high growth rate allows fast utilization of resources after resource renewal, whereas high yield can lead to larger biomass when resources are utilized and thus possibly a larger proportion of descendants can utilize the next resource pulse. In addition to growth and yield measurements at constant temperatures, we also measured these growth parameters under fluctuating temperatures that closely matched the conditions of the initial fluctuating temperature treatments. With this design, it was possible to determine whether tolerance curves change as a result of selection at fluctuating temperatures or if fluctuating environments induces specific adaptations that are not visible in tolerance curves measured in constant environments.

Materials and methods

Evolution experiment

The long-term evolution experiment was initiated from an overnight culture of a single clone of *S. marcescens* DB 11 strain (Flyg *et al.*, 1980), in DM25 medium (Lenski, 1988), supplemented with 25 mg L^{-1} of glucose. Prior to the experiment, this clone was habituated to experimental conditions by rearing it for 2 weeks in 31 °C, with a resource renewal cycle matching the experimental set-up. Experimental microcosms were wells of Bioscreen C 100 well plates (volume: 400 μL). To initiate the experiment, the overnight culture of an ancestor clone was seeded to three Bioscreen C 100 well plates, 10 populations on each plate. The plates were placed in three different thermal regimes: one in constant 31 °C, one in fluctuating temperatures with cycles of 24–31–38 °C (smooth fluctuations) and one in fluctuating temperatures cycling between 24 and 38 °C (abrupt fluctuations). The mean temperatures in the fluctuating treatments matched the mean temperature in the constant temperature treatment. In the case of smooth fluctuations, the temperature changed every 30 min, whereas for abrupt fluctuations, the temperature changed every 45 min. The temperature time series were created using programmable incubators (Lab Companion, ILP-12; Jeio Tech, Seoul, Korea). Note that in both of the fluctuating temperature treatments, the temperatures fluctuate most of the time within generations. Therefore, there is only a short time window when population growth is at maximum, and temperature is at optimum ca. 30 °C. This is the only opportunity when environmental fluctuations could theoretically occur between generations (fastest doubling time is ca. 40 min). However, the theoretical number of generations within renewal period in all evolutionary treatments is estimated to be ca. 5.32 generations [$\log_2$ (400 μL per 10 μL)] (Bennett & Lenski, 1993), which means that fluctuations occur clearly within generations. The experimental populations were transferred to new resources every 48 h by transferring 10 μL of population to the new well filled with 400 μL of DM25 medium. The experiment consisted of 27 renewals, that is it was 54 days in duration.

Isolation and measurement of the clones

After the experiment, we extracted 12 clones from each population by dilution-plating samples from every population. After 48 h of growth on DM25 agar plates, 12 clones from each population were picked from agar plates and added to 1 mL of DM 25 (each clone separately) and grown overnight. The clones were frozen in a 1 : 1 ratio with 80% glycerol, in randomized order, in four Bioscreen C 100 well plates and stored at −80 °C for growth measurements. We initiated growth measurements by cryo-replicating (Duetz *et al.*, 2000) the frozen clones to DM25 filled Bioscreen C 100 well plates. After 24 h, 10 μL of each clone culture was transferred to new plates containing DM25 and the plates were placed in Bioscreen spectrophotometers (Growth Curves Ab Ltd, Helsinki, Finland) at the desired temperature for 3–4 days, that is until growth in all wells stopped. To quantify whether evolution had

targeted tolerance curve properties in constant environments, we measured the growth of clones at seven constant temperatures: at 20.5, 24, 27.5, 31, 34.5, 38 and 41.5 °C. A temperature of 41.5 °C was lethal to all of the clones, and thus measurements at this temperature are not considered hereafter. Note that even without 41.5 °C, these temperatures cover the full range of temperatures (24–38 °C) that the clones experienced during the experiment. Thus, this thermal range is adequate in testing whether fluctuations in temperature lead to changes in tolerances to the experienced temperatures. To quantify whether the clones evolved to tolerate temperature fluctuations, they were grown at fluctuating temperatures in Bioscreen spectrophotometers continuously following the biomass growth (cycling every 30 min between 24 and 38 °C) for 3 days. Lids were not used to cover plates during this measurement due to vapour from cycles of heating and cooling. With this measurement system, there are two potential problems: cross-contamination and evaporation of medium from the wells. However, the cross-contamination risk was tested by observing bacterial growth in control wells filled with sterile medium between wells filled with nonsterile medium. During the 3 days of experiment, no growth was found in any of the control wells. Moreover, possible cross-contamination via vapour is always overwhelmed by the large number of experimental cells introduced (ca. 10 μL of high density stationary phase inoculum) to the medium by cryo-replication. Thus, the growth is always expected to reflect the growth of target cells. Although vapour somewhat reduces the volume of the medium within 3 days, randomization of clones to the measurement plates prevents the existence of systematic differences between the evolutionary treatments due to different levels of evaporation or cross-contamination in the plates. However, despite efforts to minimize contamination and evaporation, parameters between measurements at constant and fluctuating temperatures might not be directly comparable. Throughout the analyses, we used raw optical density (OD) values instead of calibrating them to cell numbers. As experimental evolution in different environments could lead to differences in cell sizes, the deduction of evolved ability to convert resources to biomass in different environments is more accurately performed with raw OD values. Therefore, our fitness measurements (growth rate and yield) correspond to the speed of biomass production and amount of biomass that bacteria can produce from limited resources, regardless of the cell size.

Statistical analyses

The growth data of the clones (absorbance at 420–580 nm, measured every 5 min) were analysed first with a MATLAB (version: 2008b; Math works Inc, Natic, MA, USA) script written by TK, for calculating maximal growth rate and yield. Maximal growth rate was determined from OD data using a 25 data point sliding window across the data and fitting linear regressions of time against log-transformed OD. Maximum growth is found by finding the steepest linear regression from sliding windows, as log transformation linearizes the exponential growth. The yield is denoted by the maximal average OD found from sliding windows. Maximal growth rate and yield for each of the clones, from each of the measured temperatures, were statistically analysed with REML mixed models in SPSS (IBM-SPSS v. 20; Chicago, IL, USA). The model included inoculum size as a fixed covariate (the average of the OD of the first three measurement points), and measurement temperature, evolutionary treatment and their interaction were modelled as fixed effects. The identity of the population was modelled as a random effect, nested within the evolutionary treatment, to control for the nonindependency of the clonal observations from the same population. Measurements at fluctuating temperatures were analysed by a model with evolutionary treatment as a fixed factor, inoculum as a fixed covariate and population identity as a random factor.

Results

When growth rate (OD 420–580 nm h^{-1}) was measured in several constant temperatures, we found that the average performance of strains from different evolutionary treatments did not differ (Table 1). However, we found a significant interaction between evolutionary treatment and measurement temperature (Table 1a, Fig. 1a). More specifically, at 27.5 °C, the clones evolved in the constant environment grew faster than the clones from smooth fluctuations ($P = 0.011$) and from abrupt fluctuations ($P = 0.028$). However, the clones from the two fluctuating environments grew at equal rates ($P = 0.721$). At 31.0 °C, the clones from the constant environment grew faster than the clones from smooth fluctuations ($P = 0.030$) and tentatively faster than clones from abrupt fluctuations ($P = 0.079$). Again, no differences in growth were found between the clones from the two different fluctuating regimes ($P = 0.675$). In addition, we found that at 38.0 °C, the clones from smooth fluctuations had a lower growth rate than clones from abrupt fluctuations ($P = 0.013$) and also had tentatively slower growth than clones from the constant environment ($P = 0.072$). At other temperatures, the growth rates of clones from different evolutionary treatments were comparable (data not shown). Population identity had a significant effect on average growth rate (Table 1). If inoculum was small, a larger growth rate followed ($b = -2.368$, SE: 0.158, $P < 0.001$).

We found that the evolutionary treatments affected average yield (OD 420–580 nm) across measurement

Table 1 Mixed model (REML) results testing the effects of evolution in constant or fluctuating environments (evolutionary treatment) on tolerance across different constant environments (constant temperatures) or on tolerance to temperature fluctuations (fluctuating temperatures). The performance was measured with maximal growth rate and biomass yield. Model included also random effect of population identity to control for the nonindependency of the clones due to shared replicate population (12 clones per population). Moreover, inoculum size was used to control for the differences in growth estimates caused by inoculum size. Note that mixed model for growth rate in fluctuating environment had convergence problems, and thus, the result from equivalent ANOVA model is presented (see more details in Materials and methods).

	Growth rate			Yield		
	F	d.f.1, d.f.2	*P*	*F*	d.f.1, d.f.2	*P*
Constant temperatures						
Evolutionary treatment	1.139	2,26.977	0.335	3.421	2,26.935	0.047
Temperature	561.060	5,2114.329	< 0.001	604.064	5,2114.049	< 0.001
Evolution × Temperature	2.423	10,2114.941	0.007	0.897	10,2114	0.535
Inoculum	229.006	1,2140.123	< 0.001	848.944	1,2126.275	< 0.001
Population	Wald Z: 2.861		0.004	Wald Z: 3.410		0.001
Fluctuating temperature						
Evolutionary treatment	11.531	2,27.33	< 0.001	2.136	2,26.185	0.138
Inoculum	35.971	1,329	< 0.001	25.106	1,345.466	< 0.001
Population	0.998	27,329	0.470	Wald Z: 2.269		0.023

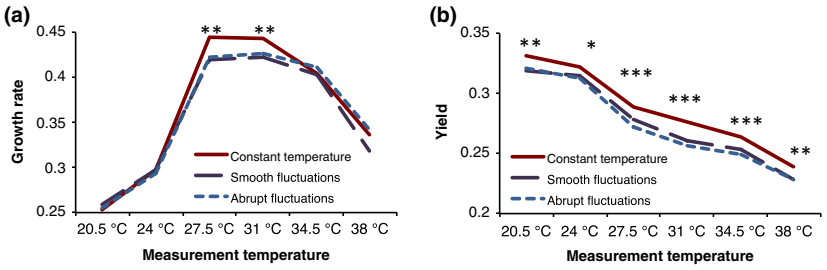

Fig. 1 (a) Maximal growth rate (OD 420–580 nm h^{-1}) and (b) yield (OD 420–580 nm) of clones evolved in constant, smoothly or abruptly fluctuating temperatures, measured at six constant temperatures. In growth rate, the clones from constant temperature outperformed the clones from both fluctuating temperatures at 27.5 °C. Same result was also evident, but to a lesser extent at 31 °C. At 38 °C, the clones from smooth fluctuations had lower growth rate than the clones from abrupt fluctuations. However, at 38 °C, the clones from either of the fluctuating environments did not differ from the clones from constant environment. In yield, the clones from constant environment always outperformed the clones from fluctuating environments (see Results for exact statistics). Statistical significance of differences due to treatments within each temperature is marked with asterisks, *, **, ***, $P = 0.05$, 0.01 and 0.001, respectively.

temperatures (Table 1, Fig. 1b). In particular, clones from constant environments (yield: 0.287; SE: 0.004) had a larger yield when compared with clones from abrupt fluctuations (yield: 0.273; SE: 0.004, $P = 0.021$). Similarly, the clones from smooth fluctuations (yield: 0.276; SE: 0.004) also had a tendency to produce a lower yield than the clones from constant environments ($P = 0.051$). The yields of the clones from the two fluctuating regimes were comparable ($P = 0.656$). There was no indication of evolutionary treatment by measurement temperature interaction on yield (Table 1). However, population identity (Table 1) and inoculum ($b = 2.551$, SE: 0.074, $P < 0.001$) had a significant effect on biomass yield.

In the analysis of growth rate during thermal fluctuations, the estimate for the identity of the population converged to zero (i.e. population effect is very small) which biases the main effects. To give a less biased estimate for this particular trait, we report results from a nested ANCOVA (Table 1, Fig. 2a), which confirmed the small and nonsignificant population effect. In pairwise tests between treatment levels, we found that clones from constant environments had clearly lower growth rate than the clones from abrupt fluctuations ($P < 0.001$) or the clones from smooth fluctuations ($P < 0.001$). Growth rates of the clones from the two fluctuating regimes were comparable ($P = 0.910$). In the ANCOVA test (Table 1), larger inoculum volume was associated with a slower growth rate ($b = -2.349$, SE: 0.392, $P < 0.001$). When inoculum-corrected growth rate (residuals from regression of growth rate on inoculum), averaged within populations with mixed model, was analysed, the same result was obtained (evolutionary treatment: $F_{2,27} = 6.685$, $P = 0.004$).

According to this analysis, the populations from a constant environment clearly grew slower than the populations from abrupt ($P = 0.007$) and smooth fluctuations ($P = 0.002$). There was no difference between growth of the clones from abrupt and smooth fluctuations ($P = 0.531$). Thus, regardless of the test, the biological interpretation of the results is unchanged.

Yield measured in fluctuating environment was not explained by evolutionary treatment (Fig. 2b) and accordingly all pairwise comparisons were also tentative or nonsignificant (constant vs. smooth fluctuations: $P = 0.054$, constant vs. abrupt fluctuations $P = 0.151$, smooth vs. abrupt fluctuations: $P = 0.611$). Moreover, we found a strong effect of population identity (Table 1.) and larger inoculum upon generating larger yields ($b = 0.794$, SE: 0.158, $P < 0.001$).

When equivalent mixed models as above were performed with ancestor clones in the model, the ancestors grew faster than the experimental strains in nearly all of the constant and fluctuating temperatures [in all pairwise (within temperatures) comparisons $P < 0.001$, ancestors growth rate at fluctuating: 0.497 (SE: 0.021), 20.5 °C: 0.355 (0.022), 23 °C: 0.396 (0.022), 27.5 °C: 0.628 (0.021) 31 °C: 0.647 (0.020)]. The only exceptions to this pattern were at 34.5 °C where the growth advantage of the ancestor, over the evolved clones, was more moderate [$P < 0.05$, in all pairwise comparisons. Ancestor's growth rate: 0.474 (0.022)]. At 38 °C, the ancestor growth rate was comparable to all evolutionary treatments [differences between growth rates: ancestor − constant: −0.008, $P = 0.739$, ancestor − smooth 0.009, $P = 0.689$, ancestor − abrupt: −0.015, $P = 0.521$. Ancestor's growth rate: 0.340 (0.022)].

Ancestor clones had a higher yield than the clones from evolutionary treatments at all constant temperatures 20.5 °C: 0.377 (SE: 0.014), 23 °C: 0.367 (0.014), 27.5 °C: 0.331 (0.013), 31 °C: 0.301 (0.013), 34 °C: 0.293 (0.014), except at 38 °C: 0.227 (0.014). At 38 °C, the yields of evolved clones and ancestors were comparable (differences between yields: ancestor − constant: −0.002, $P = 0.898$, ancestor − smooth: 0.008, $P = 0.591$, ancestor − abrupt: 0.008, $P = 0.572$). Moreover, at fluctuating temperatures, ancestors did not differ from evolved clones (differences between yields: ancestor − constant: −0.024, $P = 0.094$, ancestor − smooth: −0.013, $P = 0.371$, ancestor − abrupt: −0.015, $P = 0.296$).

Discussion

The performance of a species or a genotype in a fluctuating environment is expected to be predictable from the broadness (good performance in extremes) or elevation of tolerance curves (Levins, 1968; Lynch & Gabriel, 1987; Scheiner & Yampolsky, 1998; Condon *et al.*, 2014). However, when measured at six constant temperatures, *S. marcescens* clones that had evolved in fluctuating environments had lowered growth rates at optimum temperatures (27.5, and 31 °C), and an overall lower yield, in comparison to the clones that had evolved in constant environments. Based on expectations, such results would be interpreted to indicate a lowered ability of clones from fluctuating environments to withstand any environment, let alone fluctuating ones. However, when tolerance to thermal transitions was quantified at fluctuating temperatures, it was evident that clones that had evolved in fluctuating environments had higher growth rates than the clones that had evolved in constant environments.

Our results are clearly at odds with the theories that expect improved tolerance to extreme temperatures to evolve in fluctuating environments (Levins, 1968; Lynch & Gabriel, 1987), or with observations suggesting that fluctuating temperatures primarily select for overall higher thermal tolerance curves (Scheiner & Yampolsky, 1998; Ketola *et al.*, 2013, 2014; Condon *et al.*, 2014). Thus, the evidence from measurements taken in constant environment, when contrasted to the theories and previous work, appears only to indicate that the clones from fluctuating environments would perform poorly in fluctuating environments. Thus, within the range of temperatures measured (20.5–38 °C) or within the range of temperatures experienced during the experiment (24–38 °C, i.e. the only evolutionarily meaningful temperature range for these clones), the

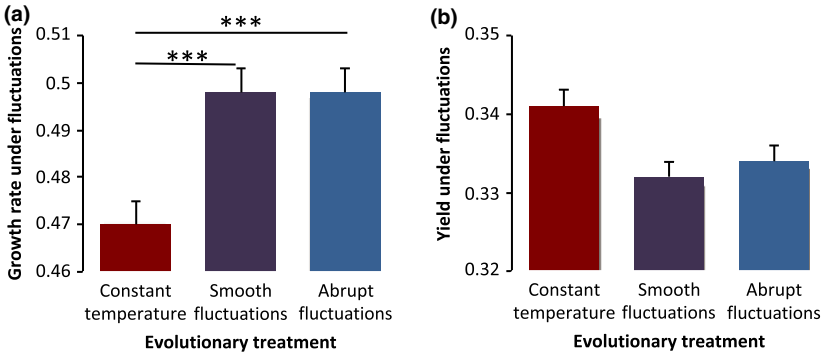

Fig. 2 Effects of evolution at constant or fluctuating temperatures on (a) maximal growth rate (OD 420–580 nm h^{-1}) and (b) yield measured under thermal fluctuations. Statistically different growth rates are marked with asterisks, ***, $P = 0.05$, 0.01 and 0.001, respectively (see Results for exact statistics).

clones adapted to fluctuating temperatures were clearly not excelling the clones adapted to constant environments. Note also that the differences in growth at high temperatures indicated only differences between the clones from abrupt and smooth fluctuations, and not between the clones from fluctuating environments and constant environment.

Most of the studies that explore evolution in fluctuating environments have concentrated on mapping growth differences in different constant environments (Kassen, 2002; but see: Leroi *et al.*, 1994; Kassen & Bell, 1998; Hughes *et al.*, 2007). However, selection for coping with fluctuating environments can also target traits that are not visible in tolerance curves measured in constant environments. In accordance with this idea, the clones from both of the fluctuating environments developed improved growth at fluctuating temperatures (Fig. 2a). The clearly increased ability to grow quickly under thermal fluctuations could be explained by the evolution of traits responsible for acclimation and inducible phenotypic plasticity, such as heat-shock protein expression at extreme temperatures (Sorensen *et al.*, 2003; Ketola *et al.*, 2004). Alternatively, selection could have led to performance specialization: an improved utilization of the short time window of optimal conditions after exposure to extreme conditions (Gilchrist, 1995; New *et al.*, 2014) or that clones have evolved lower responsiveness to extreme environments altogether (Suiter *et al.*, 2003; see also, Kawecki, 2000). When tolerance to fluctuations was measured in yield during fluctuations, the clones from different evolutionary treatments were comparable. Interestingly, it seems that in tolerance curve literature, no one has previously suggested that adaptation to fluctuating environments could be negatively reflected in tolerance curves (Leroi *et al.*, 1994; Kassen & Bell, 1998; Hughes *et al.*, 2007). Such a result is exactly what would be expected if there is a trade-off between adaptation to fluctuating and constant environments, so that adaptation to one kind of environment negatively affects the ability to perform in the other environment [i.e. 'local adaptation' to temperature fluctuations and absence of them (Savolainen *et al.*, 2013)].

Theories on environmental adaptation frequently predict that evolution under fast environmental fluctuations (i.e. within generation fluctuations) should lead to generalist genotypes (Levins, 1968; Lynch & Gabriel, 1987), with better tolerance across all environments or increased tolerance at extreme environments. When we tested this hypothesis in conditions where bacteria experienced fluctuations within generations, evolution led to reductions in fitness of fluctuation-adapted clones when the tolerance was measured in constant environments. However, these seemingly suboptimal clones clearly outperformed the clones that evolved in a constant environment when the performance was measured by maximal growth rate in fluctuating environments. Thus, against the widespread belief that measurements taken in constant environments can also describe adaptation to fluctuating environments (Helmuth *et al.*, 2005; Deutsch *et al.*, 2008; Schulte *et al.*, 2011; Huey *et al.*, 2012), we show here that the best adapted individuals for tolerating fluctuating environments could be wrongly judged inferior based on their tolerances at constant environments. In the worst case scenario, neglecting tolerance to environmental fluctuations could lead to false predictions of the fate of populations and genotypes under climate change-induced environmental fluctuations.

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