doi:10.1111/evo.12148

# FLUCTUATING TEMPERATURE LEADS TO EVOLUTION OF THERMAL GENERALISM AND PREADAPTATION TO NOVEL ENVIRONMENTS

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Received June 20, 2012
Accepted April 11, 2013
*Data Archived: Dryad doi:10.5061/dryad.2k4vc*

**Environmental fluctuations can select for generalism, which is also hypothesized to increase organisms' ability to invade novel environments. Here, we show that across a range of temperatures, opportunistic bacterial pathogen *Serratia marcescens* that evolved in fluctuating temperature (daily variation between 24°C and 38°C, mean 31°C) outperforms the strains that evolved in constant temperature (31°C). The growth advantage was also evident in novel environments in the presence of parasitic viruses and predatory protozoans, but less clear in the presence of stressful chemicals. Adaptation to fluctuating temperature also led to reduced virulence in *Drosophila melanogaster* host, which suggests that generalism can still be costly in terms of reduced fitness in other ecological contexts. While supporting the hypothesis that evolution of generalism is coupled with tolerance to several novel environments, our results also suggest that thermal fluctuations driven by the climate change could affect both species' invasiveness and virulence.**

**KEY WORDS:** **Bacteriophage, *Drosophila melanogaster*, host, invasion, oxidative stress, PPV, predation, *Serratia marcescens*, *Tetrahymena thermophila*, virulence, virus..**

Environmental fluctuations have been suggested to select for genotypes that are capable of performing well across a wide range of different environments (e.g., Levins 1968; Lynch and Gabriel 1987; Gomulkiewicz and Kirkpatrick 1992; Scheiner 1993; Kassen 2002). This evolved tolerance could also decrease or increase the ability to inhabit other, novel, environments (e.g., Huey and Hertz 1984; Hoffmann and Parsons 1993a,b; Cullum et al. 2001; Bubliy and Loeschcke 2005). Thus, in some cases, preadaptation to a novel environment could play a key role in species invasions and in the emergence of new pathogens (Arnold et al. 2007; Lee and Gelembiuk 2008).

Environmental fluctuations have the potential to cause several changes in populations and individuals. For example, fluctuating selection could maintain genetic variation and polymorphism (Levene 1953; MacKay 1980, see also Kassen 2002) and potentially lead to divergent evolution between populations (Cooper and Lenski 2010). Moreover, rapidly and especially unpredictably fluctuating environments could select for reversible phenotypic plasticity and bet-hedging (e.g., Cohen 1966; Arnoldini et al. 2012). However, most of the theoretical work has concentrated on the changes in tolerance curve: fluctuating environments are suggested to select for individuals with increased tolerance over





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INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

the most frequently experienced conditions, as their tolerance curve width evolves to match the width of the environmental fluctuations (Levins 1968; Lynch and Gabriel 1987; Gomulkiewicz and Kirkpatrick 1992; Scheiner 1993; Kassen 2002). However, the outcome of evolution might be affected by the frequency of environmental fluctuations relative to organism's generation time; depending on theoretical assumptions, generalism might be more likely to evolve if the environmental fluctuations occur within generation (Lynch and Gabriel 1987) or if it happens between generations (Levins 1968, p. 20; Gilchrist 1995). Yet, many of the experimental studies to date demonstrate that generalism emerges when populations are evolving in fluctuating environments regardless of the frequency of the variation (reviewed in Kassen 2002 and Buckling et al. 2006, but see: Jasmin and Kassen 2007). Despite the several attempts to test evolution of generalism, only few studies have addressed the impact of fluctuating temperatures on evolution of thermal tolerance (Bennet and Lenski 1993; Ketola et al. 2004; Duncan et al. 2011). Testing evolution of generalism in fluctuating temperatures is important considering the predictions of climate change scenarios that predict not only increased mean temperatures but also increased thermal variation (IPCC 2007).

Adaptation to environmental fluctuations (e.g., temperature fluctuations) can also lead to general environmental tolerance. For example, expression of heat shock proteins, which have been associated with resistance against several kinds of stresses (reviewed in Sørensen et al. 2003), can evolve as a response to fluctuating temperature (Ketola et al. 2004). Moreover, growing and reproducing in a varying environment can be energetically expensive, and therefore, selection could simply favor energetic efficiency and better growth or viability (van Noordwijk and de Jong 1986; Parsons 1990, 2005; Scheiner and Yampolsky 1998; see also Whitlock 1996). This, in turn, can translate directly to increased tolerance to any kind of stressful environment. However, adapting to fluctuating environment might also be costly. For example, evolution of broad tolerance could lead to lowered performance in optimal environments (e.g., Levins 1968; Huey and Kingsolver 1993). Alternatively, the costs of changed tolerance could correlate negatively with fitness in some other ecological context in some other environment (Huey and Hertz 1984; Friman et al. 2009). In addition, the lowered fitness in other environments could also result from mutation accumulation on characters that are not used in fluctuating environment (Whitlock 1996; Hall and Colegrave 2008; Mikonranta et al. 2012). The "hidden" fitness costs, evident only in different ecological contexts, could potentially explain why the cost of generalism is not often found in the selective environment (reviewed in Kassen 2002).

Thus, evolution in fluctuating environments could also be an important determinant for population's performance when species are exposed to a novel environment, either due to changes in local conditions, or because of introduction to a new habitat. Interestingly, evidence from the wild suggests that the source areas of invasive species are often characterized by disturbance. For example, this seems to hold for weedy plants, invasive argentine ants experiencing repeated flooding in their native areas, and for species that invaded the Great Lakes from disturbed habitats of the old world (reviewed in Lee and Gelembiuk 2008). Evolution of species invasiveness is also analogous to the emergence of new virulent microorganisms. There is growing evidence that a range of ecological and environmental factors such as predation or temperature can affect the virulence of opportunistic pathogens (e.g., Brüssow 2007; Casadevall and Pirofski 2007; Friman et al., 2009, 2011; Barrett et al. 2011; Mikonranta et al. 2012). Specifically, Arnold et al. (2007) have suggested that environmental fluctuations play an important role in preselecting virulence traits of bacterial pathogens. Despite the indirect evidence of correlative changes with invasiveness and virulence, there is no study explicitly testing if environmental fluctuations concurrently select for generalism, invasiveness (i.e., general tolerance across many different environments), and virulence.

Here, we set up a replicated evolution experiment where populations of a common, broad-spectrum opportunistic bacterial pathogen *Serratia marcescens* were propagated under constant or daily fluctuating temperatures for about 1000 generations. First, we tested if clones from populations experiencing fluctuating temperature had evolved temperature generalism (e.g., Levins 1968; Huey and Kingsolver 1993). Second, we tested how evolution in fluctuating versus constant environment affected the growth and yield of the bacteria in several novel environments including the presence of chemicals, the presence of natural microbial enemies (ciliate predator *Tetrahymena thermophila* and lytic bacteriophage PPV (*Podoviridae*; Friman et al. 2011). Moreover, we tested if virulence in its natural host *Drosophila melanogaster* (Flyg et al. 1980) had changed because of fluctuating environments. Finally, we tested if selection by fluctuating environments leads to more divergent evolution between replicate populations (Cooper and Lenski 2010).

## Methods

*Serratia marcescens* bacterium (ATCC 13880) is a broad-spectrum environmental pathogen inhabiting many freshwater, marine, and soil environments (Grimont and Grimont 1978). It opportunistically infects multicellular organisms such as plants, corals, nematodes, insects, fish, and mammals including immunocompromised humans (e.g., Grimont and Grimont 1978; Patterson et al. 2002; Mahlen 2011). To minimize the effects of past evolutionary history, *S. marcescens* bacterium was reared in constant 25°C for 3 weeks with similar resource conditions (below) as in the main selection experiment.

## EXPERIMENTAL EVOLUTION

We initiated the evolution experiment from a single *S. marcescens* colony (i.e., the genetic diversity was zero) that was grown overnight to high density. Twenty replicate populations were established on two 100-well spectrophotometer plates (Growth Curves Ltd., Helsinki, Finland) that were assigned to either stable (10 populations) or fluctuating (10 populations) temperatures. We used two temperature-controlled spectrophotometers (Bioscreen C, Growth Curves Ltd..) to create the following temperature selection regimes: constant 31°C, and daily fluctuating (from 24°C to 38°C, mean 31°C). These temperature selection regimes were chosen on the basis of preliminary tests of thermal tolerance with *S. marcescens*, and one-day fluctuation is comparable to frequency of fluctuations in Bennet and Lenski (1993). Replicated *S. marcescens* populations were propagated throughout the experiment in phosphate buffered pH 7.5 cereal leaf extract medium with final concentration of 2.15 mg/L plant detritus (medium described in Friman et al. 2008). Every 24 h (±1 h) and approximately every 45 generations, 40 μL of the populations were transferred into new wells filled with 360 μL of fresh medium, and returned to the spectrophotometer to keep populations in constantly growing phase. Every fourth day we changed the plates between the two machines and set temperatures to match experimental treatments to exclude confounding effects of spectrophotometer identity.

## ISOLATION OF CLONES AND CONSTRUCTION OF CLONE LIBRARIES

After 3 weeks (approximately 1000 generations), we isolated 12 colonies from each replicate population by serial dilution and plating. The clones were isolated after 48 h of growth on nutrient broth plates (2.5 g yeast extract, 10 g nutrient broth, and 15 g agar in 1 L $dH_2O$), by randomly picking 12 colonies from single plate. The isolated clones were grown for 24 h at 31°C in liquid medium and stored in −80°C in 50% glycerol. To prepare clonal libraries that allow rapid and efficient measurement protocols, all clones were thawed and regrown at 31°C for 48 h. Subsequently, 400 μL inoculums were supplemented with 50% glycerol, transferred to randomized locations on four 100-well Bioscreen plates, and frozen (−80°C) for later measurements. With randomization, we ascertained that every plate contained an equal number of clones from each population to ensure a balanced setup for statistical testing.

## MEASUREMENT OF GROWTH IN DIFFERENT ENVIRONMENTS

The frozen clones were transferred onto new plates prefilled with 400 μL of medium with heat-sterilized cryoreplicators (Enzyscreen B.V., Haarlem, The Netherlands; Duetz et al. 2000), to standardize the growth conditions between clones from different thermal regimes, and to remove glycerol residue potentially affecting bacterial growth. After 24 h growth at 31°C, bacterial suspensions were replicated on Bioscreen measurement plates that contained either chemical or biological stressor. Clones' performance was measured in one environmental condition at a time.

We measured growth of all 240 clones for 48 h at 24°C, 31°C, and 38°C in medium without or with DTT (dithriotreitol 1 mg/mL, at 31°C) or $H_2O_2$ (hydrogen peroxide 0.001%, at 31°C). These chemicals have previously been used to test bacterial ability to tolerate problems in redox balance (DTT) or oxidizing stress ($H_2O_2$; Cullum et al. 2001; Kishony and Leibler 2003). We measured the effect of predation on each clone's growth by adding about 250 cells of particle-feeding ciliate *T. thermophila* (strain ATCC 30008) to the bacterial suspension, and followed bacterial growth at 31°C for several days. *Tetrahymena thermophila* readily preys on *S. marcescens* (Friman et al. 2008). Before transferring *T. thermophila* cells into the bacterial solution, the predators' own proteose peptone growth medium (10 g proteose peptone, 25.5 g yeast extract in 1 L $dH_2O$) was washed away by centrifuging the cell culture at 768 × *g* for 8 min and resuspending the cell pellet to $dH_2O$. Washing was repeated twice. We measured the effect of lytic bacteriophages (PPV) on bacterial growth similarly (at 31°C), adding about $10^4$ phage particles to bacterial suspension prior to measuring the growth. PPV stock was prepared as follows. LB-Soft agar (0.7%) from semiconfluent plates was collected and mixed with LB (4 mL per plate), and incubated for 3.5 h at 37°C with aeration (LB: 10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L $H_2O$, pH 7.5 adjusted with NaOH). Debris was removed by centrifugation for 20 min at 9682 × *g* at 5°C. Stock was filtered with 0.2 μm Acrodisc® Syringe Filters (Pall, Port Washington, NY) and diluted 1:1000 in $dH_2O$ to give a stock with approximately $2.0 \times 10^6$ pfu/mL. When associated with the PPV virus, bacteria first start growing and growth is affected by the presence of viruses. After viruses infect enough bacteria, the bacterial density suddenly drops. After a while the bacterial population regains its growth ability. Because we focused on how novel environments affect growth at first encounter, we used the data that preceded the population collapse.

## GROWTH PARAMETERS

Maximum growth rates were determined from biomass growth data recorded for 96 h at 5 min intervals with two spectrophotometers (absorbance at 420–580 nm, wide band option). We estimated the growth rate with a MATLAB (version 2008b; MathWorks Inc., Natick, MA) script that fits linear regressions into ln-transformed population growth data consisting of 30 datapoints' sliding time window. Medium background absorbance was subtracted from the data prior to the analysis. The time window with maximum slope was determined as the maximum growth rate. Maximum population size (yield) was determined similarly as the maximal arithmetic mean optical density value in the sliding window data.

**Table 1.** Analysis of variance (ANOVA) results on the effects of variable or stable temperature on the evolution of maximal growth rate (panel A) and yield (panel B) in *Serratia marcescens* in various environments. First analysis tests thermal adaptation across three temperatures (24°C, 31°C, and 38°C). The second analysis tests the effects of evolutionary changes in novel and stressful environments (DTT, $H_2O_2$, with *Tetrahymena thermophila* predatory ciliate, or with lytic virus PPV). Last test includes the full dataset containing all measurement environments.

| | Temperature environments | | | Novel environments | | | All environments | | |
|---|---|---|---|---|---|---|---|---|---|
| | *F* | df1, df2 | *P* | *F* | df1, df2 | *P* | *F* | df1, df2 | *P* |
| (A) Growth rate | | | | | | | | | |
| Evolution treatment | 88.510 | 1,18.108 | <0.001 | 14.264 | 1,18.178 | 0.001 | 42.029 | 1,18.113 | <0.001 |
| Environment | 472.414 | 2,692 | <0.001 | 212.211 | 3,929 | <0.001 | 280.030 | 6,1640 | <0.001 |
| Evolution treatment by environment | 2.992 | 2,692 | 0.051 | 0.248 | 3,929 | 0.863 | 0.808 | 6,1640 | 0.564 |
| Population (evolution treatment) | 0.951 | 18,692 | 0.516 | 0.545 | 18,929 | 0.545 | 1.163 | 18,1640 | 0.284 |
| (B) Yield | | | | | | | | | |
| Evolution treatment | 7.074 | 1,18.11 | 0.016 | 6.390 | 1,18.082 | 0.021 | 9.160 | 1,18.065 | 0.007 |
| Environment | 222.939 | 2,692 | <0.001 | 389.984 | 3,929 | <0.001 | 455.204 | 6,1640 | <0.001 |
| Evolution treatment by environment | 0.119 | 2,692 | 0.888 | 0.215 | 3,929 | 0.886 | 0.112 | 6,1640 | 0.995 |
| Population (evolution treatment) | 0.910 | 18,692 | 0.567 | 2.023 | 18,929 | 0.007 | 2.010 | 18,1640 | 0.007 |

## MEASURING BACTERIAL VIRULENCE

The virulence assay was modified from Nehme et al. (2007). After culturing bacteria for 24 h in LB medium at 31°C, we mixed 800 μL of bacterial suspension with 800 μL of 100 mM sucrose. This solution was absorbed into a cotton dental roll (Top Dent, Lifco Dental, Enköping, Sweden) folded on the bottom of a standard 75 × 23 mm fly vial (Sarstedt, Nümbrecht, Germany). Next, we transferred ten 2–3 days old *D. melanogaster* adults from a large laboratory colony (Oregon R) to each vial and sealed the vials with cotton plugs. This was done for all the 240 bacterial clones (equalling 240 vials) and the death rate of flies was monitored over next 4 days, at about 6 h intervals. During the virulence assay, the vials were held in two thermostat controlled growth chambers at 31°C (Lab Companion, ILP-02/12) and the locations of vials in the chambers, and between the chambers were changed in every 6 h. The 50 Mm sucrose solution was used to control the baseline death rate ($N = 6$). None of the control flies died during the virulence assay.

## DATA ANALYSIS

To analyze the evolutionary changes in growth rate and yield, we used univariate analysis of variance (ANOVA). First, we analyzed if there had been evolution of thermal generalism by fitting growth rate and yield as dependent variables, and temperature treatment (stable/fluctuating) and the measuring temperature (24°C, 31°C, and 38°C) as fixed factors. Second, to explore the effect of thermally induced evolution on bacterial growth and yield in different novel environments, we ran ANOVAs with the measuring environment (DTT, $H_2O_2$, protist predation, and viral parasitism) and the temperature treatment as fixed factors. This test is needed to explore the effects of evolution on growth in novel environments independently from thermal environments. Finally, we reanalyzed the data by including all the measurement environments in to the same analysis (Table 1). Data deposited in the Dryad repository: doi:10.5061/dryad.2k4vc.

In all of the analyses, we included all factor interactions. Moreover, to control for possible spectrophotometer-specific differences during the measurements, we included machine identity as a fixed factor nested within the measurement environment, which had significant effect on growth rate and yield estimates. To control for small differences in the concentration of bacterial inoculums, we used the mean of first three bacterial density measurements (0, 5, and 10 min) as a covariate in the analyses. In all of the analyses density of inoculum increased the growth and yield estimates ($P < 0.001$, in all tests). In addition, we fitted bacterial population identity as a random factor nested within the temperature regime, to control for the nonindependency of the clones. If the population variation is significantly different from 0, it indicates that replicate populations evolve differently (diverge) within the treatment, whereas nonsignificant population variation indicates similar (convergent) evolution between replicate populations within the treatment.

Effects of the temperature regime on bacterial virulence against *D. melanogaster* were quantified with Cox regression by

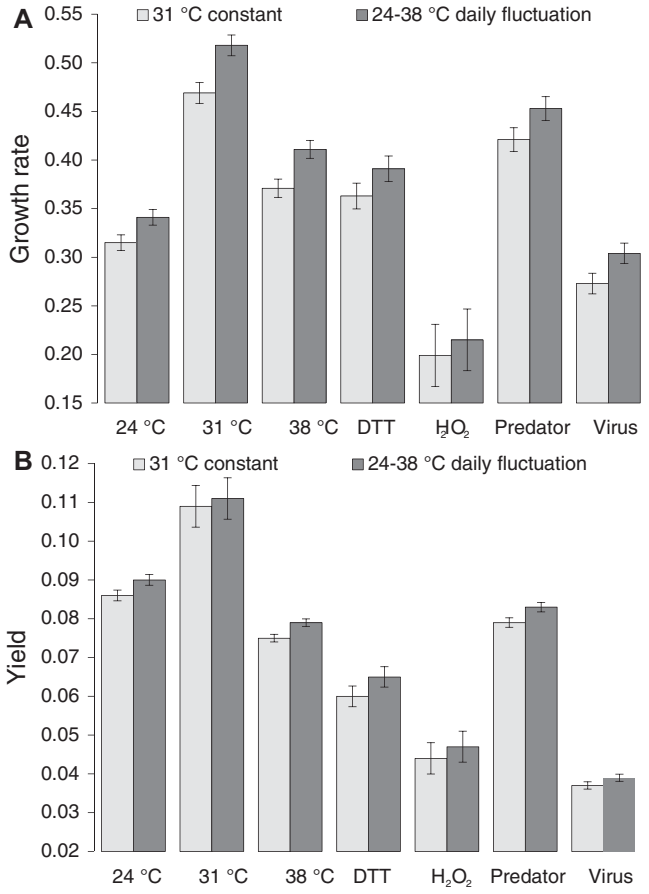


**Figure 1. (A) Average maximal growth rate (±2 standard errors) of *Serratia marcescens*—bacteria evolved either in constant 31°C or in daily fluctuating temperature (between 24°C and 38°C). (B) Average biomass yield (±2 standard errors) of *S. marcescens*—bacteria evolved either in constant 31°C or in daily fluctuating temperature (between 24°C and 38°C). Estimates correspond to estimated marginal means for treatment effects and their standard errors.**

fitting the temperature regime and population identity (20 levels) as categorical covariates. The analyses were performed with SPSS (version 19; IBM SPSS, Chicago, IL).

# Results

## EVOLUTION OF THERMAL GENERALISM

Selection by fluctuating temperature increased bacterial growth rate across all measurement temperatures compared to clones that had evolved under constant temperature (Table 1; Fig. 1A). Clones from fluctuating temperature were superior at 31°C ($F_{1,18.037} = 49.99$, $P < 0.001$) followed by 38°C ($F_{1,18.000} = 29.97$, $P < 0.001$) and 24°C ($F_{1,18.265} = 22.912$, $P < 0.001$). Moreover, the average bacterial growth rate was highest at 31°C, followed by 38°C, whereas the lowest growth rate was found at 24°C ($P < 0.001$, in all pairwise tests, Table 1; Fig. 1A).

Fluctuating temperature regime also selected for increased biomass yield compared to clones evolving in constant temperature (Table 1; Fig. 1B). Although there was no interaction between temperature regime (fluctuating vs. constant) and measurement temperature (24°C, 31°C, and 38°C; Table 1), pairwise comparisons between measurement temperatures revealed that clones from fluctuating temperature had greater yield at 24°C ($F_{1,\ 18.419} = 31.590$, $P < 0.001$) and at 38°C ($F_{1,\ 17.999} = 32.958$, $P < 0.001$), than clones from constant temperature, whereas no difference was found at 31°C ($F_{1,\ 18.044} = 0.524$, $P = 0.478$; Fig. 1B). Highest yield was found at 31°C, whereas yield in 24°C was clearly lower. Lowest yield was found at 38°C (in all pairwise tests; $P < 0.001$; Table 1; Fig. 1B).

### *Effects of thermal evolution on performance in novel environments*

Based on the growth rate, clones that evolved in fluctuating temperature were generally superior across novel environments (Table 1). This effect was clearest in the presence of virus ($F_{1,18.416} = 7.104$, $P = 0.016$) and predator ($F_{1,18.667} = 14.852$, $P < 0.001$), whereas temperature regime had only weak effect in DTT ($F_{1,18.226} = 4.020$, $P = 0.060$), or in $H_2O_2$ ($F_{1,17.966} = 0.863$, $P = 0.365$). Moreover, we found that average growth rate differed in different measurement environments (all pairwise tests $P < 0.001$; Table 1; Fig. 1A).

Overall, clones from different temperature regimes did not differ in terms of yield in any of the novel environments (Table 1). However, in pairwise testing, clones from fluctuating temperature had higher yield than clones from constant temperature, when measured with virus ($F_{1,18.844} = 24.05$, $P < 0.001$) and the predator ($F_{1,18.828} = 22.23$, $P < 0.001$). However, no clear differences in yield were found when clones were grown rate in DTT ($F_{1,18.178} = 4.376$, $P = 0.051$) or $H_2O_2$ ($F_{1,17.964} = 0.553$, $P = 0.467$). Again, there were strong effects of measurement environments on average yield (all pairwise tests $P < 0.001$, except predation vs. DTT $P = 0.601$; Table 1; Fig. 1B).

### *Effects of thermal evolution on performance across all measured environments*

When all measurement environments were analyzed in the same analysis, both growth rate and yield were higher with clones that had evolved in fluctuating temperature (Table 1). No significant interaction between temperature regime and measurement environment was found. Most of the measurement environments differed in their effect on average growth and yield (most pairwise tests $P < 0.001$, but yield at 38°C vs. predator: $P = 0.555$, 38°C vs. DTT: $P = 0.601$, and DTT vs. predator: $P = 0.348$; Table 1; Fig. 1).

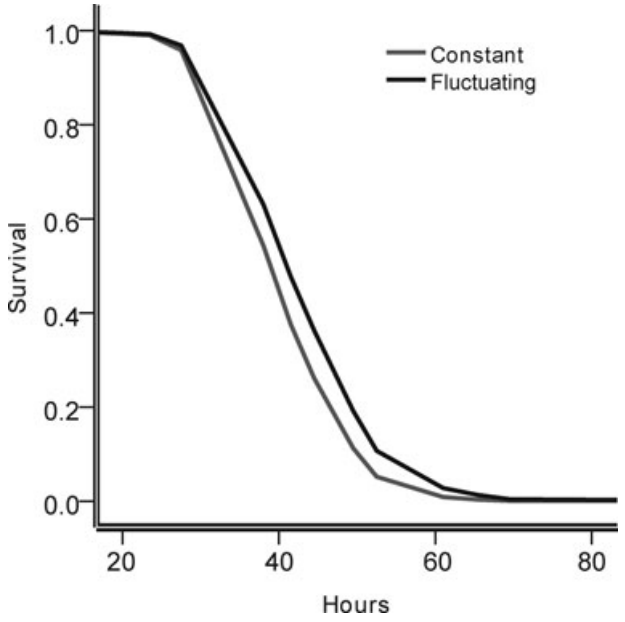


**Figure 2.** **Cumulative survival of *Drosophila melanogaster*—flies infected with clones of *Serratia marcescens* bacteria evolved either in constant 31°C or in daily fluctuating temperature (between 24°C and 38°C).**

### EFFECTS OF THERMAL EVOLUTION ON VIRULENCE

Evolution in fluctuating temperature decreased bacterial virulence in *D. melanogaster* host (Cox regression; Wald = 4.485, $P$ = 0.034; Fig. 2).

#### *Convergent or divergent evolution*

When we fitted the identity of the population (nested within temperature regimes) in analyses of growth, we found no evidence of divergent evolution between the populations (population effect was nonsignificant; Table 1A). However, in novel environments, yield was affected by the population identity suggesting some divergence of the populations (Table 1B). However, tests done for each of the novel environments separately could not pinpoint the environment that was singly responsible for the effect of the population identity (nested within temperature treatment) on yield (24°C: $F_{1,18} = 0.543$, $P = 0.935$; 31°C: $F_{1,18} = 0.685$, $P = 0.824$; 38°C: $F_{1,18} = 0.860$, $P = 0.627$; DTT: $F_{1,18} = 1.345$, $P = 0.162$; $H_2O_2$: $F_{1,18} = 1.355$, $P = 0.157$; predator: $F_{1,18} = 1.556$, $P = 0.073$; virus: $F_{1,18} = 1.429$, $P = 0.120$). Corresponding values for growth rate were: (24°C: $F_{1,18} = 0.856$, $P = 0.632$; 31°C: $F_{1,18} = 0.831$, $P = 0.684$; 38°C: $F_{1,18} = 1.162$, $P = 0.296$; DTT: $F_{1,18} = 2.011$, $P = 0.010$; $H_2O_2$: $F_{1,18} = 0.838$, $P = 0.654$; predator: $F_{1,18} = 0.576$, $P = 0.915$; virus: $F_{1,18} = 0.708$, $P = 0.801$).

Moreover, when we tested if variation due to population identity differed between the temperature regimes in yield and growth rate (resolved by separate $Z$-tests from estimated marginal means of population variation and their standard errors, estimated separately for both of the temperature regimes), we did not find evidence of higher degree of divergence of populations in fluctuating temperatures than in constant temperatures (24°C: $Z_{1,9} = 0.33$, $P = 0.580$; 31°C: $Z_{1,9 =} 0.28$, $P = 0.612$; 38°C: $Z_{1,9} = 0.01$, $P = 0.911$; DTT: $Z_{1,9} = 0.14$, $P = 0.715$; $H_2O_2$: $Z_{1,9} = 0.05$, $P = 0.835$; predator: $Z_{1,9} = 0.30$, $P = 0.599$; virus: $Z_{1,9} = 0.82$, $P = 0.389$), or in growth rate (24°C: $Z_{1,9} = 0.34$, $P = 0.573$; 31°C: $Z_{1,9} = 0.22$, $P = 0.652$; 38°C: $Z_{1,9} = 0.31$, $P = 0.593$, DTT: $Z_{1,9} = 1.26$, $P = 0.289$, $H_2O_2$: $Z_{1,9} = 0.19$, $P = 0.676$, predator: $Z_{1,9} = 0.05$, $P = 0.825$, virus: $Z_{1,9} = 0.06$, $P = 0.813$).

We found a significant population effect in virulence (Cox regression; Wald = 33.332, df = 18, $P$ = 0.015), and when the magnitude of variation due to replicate population was estimated for both temperature regimes separately, no population effect was found in the constant temperature environment (Wald = 13.426, df = 9, $P$ = 0.144), while significant difference was found in the fluctuating temperature (Wald = 20.076, df = 9, $P$ = 0.017). However, population effects (i.e., the degree of convergence) did not differ statistically between the temperature regimes (Levene's test: $F_{1,18} = 1.428$, $P = 0.248$).

## Discussion

Environmental temperature fluctuations have been suggested to select for thermal generalism (Levins 1968; Huey and Kingsolver 1993; Kassen 2002) and increase the tolerance across multiple novel environments (Huey and Hertz 1984; Hoffmann and Parsons 1993a,b; Cullum et al. 2001; Bubliy and Loeschcke 2005; Arnold et al. 2007). In this study, we present experimental evidence supporting these ideas: adaptation to fluctuating temperature led to evolution of thermal generalism that increased fitness in several novel environments compared to bacteria that had adapted to constant temperature. However, this fitness advantage was less clear in the presence of novel abiotic stressors, and the evolution of thermal generalism was costly in other ecological context in terms of attenuated virulence in *D. melanogaster* host.

### EVOLUTION OF THERMAL GENERALISM

We found that thermally fluctuating environment selected for bacterial clones that were capable to grow faster and to reach higher yield in most of the assessed temperatures compared to clones that had evolved in constant temperature conditions. As the clones from fluctuating environment had also higher yield, our results support the hypothesis that fluctuating temperature can select for true generalists (Levins 1968; Huey and Kingsolver 1993) instead of causing a life-history shift between strategies to grow fast in high resource conditions (maximum growth rate) and to grow efficiently under low resources (yield) (Velicer and Lenski 1999). Evidence for thermal generalism in the previous studies (Bennet and Lenski 1993; Leroi et al. 1994; Ketola et al. 2004; Duncan et al.

2011; for a review see Kassen 2002) has not been as evident as in our study.

Interestingly, the superiority of the clones from fluctuating temperature was clearest at optimal conditions of 31°C. This is in contrast with most theoretical predictions that often expect generalists to pay higher growth cost at optimum conditions compared to specialists (e.g., Levins 1968; Huey and Kingsolver 1993). This "Jack-of-all-trades is a master of none"——hypothesis has gained very little experimental support (reviewed in Kassen 2002). It is possible that evolving in optimal conditions without a strong selective pressure leads to accumulation of mildly deleterious mutations that affect negatively the overall growth ability across environments (Collins and Bell 2004). Instead, selection against mutations lowering the general growth ability could have been stronger in fluctuating temperature regime, leading the strain to retain high overall growth rate across different environments.

In certain cases, selection in fluctuating environment can lead to maintenance of specialist genotypes. For example, if productivity of environments is very different, evolution could lead to specialists that match the most productive environment (Jasmin and Kassen 2007). Alternatively, fluctuating environment could select individuals that are performance specialists but tolerance generalists; growing and reproducing only within a narrow range of environments but still tolerating a broad range of environments (Gilchrist 1995). Thus, our experimental lines in the fluctuating environment could have been still evolving toward specialism in the most productive/optimal environment (31°C). However, we feel that this is an unlikely scenario as our experimental lines experience only transiently 31°C, and thermal selection occurred most of the time at 24°C or at 38°C. As a result, the clear growth difference between the temperature treatments at 31°C is more likely due to the strong correlative effects on the overall thermal tolerance in response to selection experienced in the temperature extremes.

## CORRELATED EFFECTS OF THERMAL VARIATION ON PERFORMANCE IN NOVEL ENVIRONMENTS

Although the trade-off resulting from thermal generalism is often sought from decreased growth at optimal temperatures, it is also possible that the cost could be seen as a lowered performance when facing an entirely novel environment, or in a totally different, unmeasured trait (Huey and Hertz 1984). This kind of phenomenon has been observed previously, when widened bacterial host range led to lowered growth (Hall et al. 2011). In contrast with this "cost of adaptation" view, researchers have suggested the existence of general environmental tolerance (Hoffmann and Parsons 1993a,b; Bubliy and Loeschcke 2005), where selection in one environment favors mechanisms that are beneficial also in other environments. For example, Cullum et al. (2001) found tentative evidence that evolution at constant high temperature could improve *Escherichia coli*'s tolerance also in other environments, whereas Lee et al. (2012) found positive correlation between general stress tolerance and virulence in Shiga toxin-producing isolates of *E. coli* O157. Moreover, Bubliy and Loeschcke (2005) found that in *Drosophila*, selection for cold, heat, starvation, or desiccation resistance increased the flies' ability to cope with most of these environments regardless of the particular selection regime.

We found a strong effect of fluctuating temperature treatment on enhanced bacterial growth across all novel measurement environments. We conclude that the variation in growth must be mostly due to general tolerance since there was no interaction between measured environments and temperature treatment. Growth under different chemical stresses showed less clear differences between temperature treatments compared to biotic stresses. General tolerance is expected to occur in stresses that share wide range of biochemical reactions, and it could be that temperature had selected for general growth abilities rather than oxidative stress traits ($H_2O_2$), or traits associated with DTT resistance. However, overall improved growth ability could explain why fluctuating temperature preadapted bacteria to better tolerate (or grow with) both parasitic phages and protozoan predators. These enemies are major causes of bacterial mortality under natural conditions (Fenchel 1987; Jurgen and Matz 2002; Abedon 2008), and thus, are also likely to play a major role in bacterial invasion success (Elton 1958; Levine 2000; Jiang et al. 2011). Our results thus suggest that evolution of generalism could indirectly lead to emergence of highly invasive species that can survive under wide ranges of environmental conditions due to high general stress tolerance (Lee and Gelembiuk 2008).

## CORRELATED EFFECTS BETWEEN FLUCTUATING TEMPERATURE REGIME AND VIRULENCE

We found that adaptation to fluctuating temperature regime led to attenuated virulence in *D. melanogaster* insect host, which is in contrast with the previous idea of positive correlation between tolerance of environmental harshness and high virulence (Arnold et al. 2007). Virulence (i.e., the time required to kill the host) is a relatively good measure of bacterial fitness within the host (Nehme et al. 2007). Thus, decreased virulence could be seen as a cost of generalism (sensu Huey and Hertz 1984), if the less virulent genotypes lose the within-host competition to their more virulent rivals (Frank 1996). However, we found that the least virulent strains grew most effectively in liquid medium. A recent study has shown that high growth rate of *Salmonella typhimurium* strains can be coupled with low virulence due to a loss of an important virulence factor (type III secretion system), which is costly to maintain in terms of reduced growth (Sturm et al. 2011). Moreover, our findings are consistent with previous studies, showing that protozoan predation, parasitism (phages),

or temperature can lead to changes in virulence of *S. marcescens* (Friman et al. 2009, 2011; Mikonranta et al. 2012).

Fluctuating environments could also lead to divergent evolution between replicate populations, which could be interpreted as a signature of drift, weak selection, or existence of alternative mutational paths for evolution (Travisano et al. 1995; Cooper and Lenski 2010; Mikonranta et al. 2012). However, no support for this claim was found, as the magnitude of the variation explained by the population identity was similar when it was compared between temperature regimes. Traits that have weaker impact on fitness could also be more prone to divergent evolution. We found that across all measured environments, yield indicated a significant signature of divergence (statistically significant population effect; Table 1B). However, we could not pinpoint this population effect to any particular environment, which suggests that the population divergence occurred on yield in general. It is noteworthy that growth rate in DTT, and virulence indicated significant population divergence, suggesting that these traits might be more weakly selected in both of our selection regimes. Moreover, when between-populations variation was analyzed separately for virulence, the divergent evolution was evident in the fluctuating temperature regime but not in the constant one. Thus, lower virulence in fluctuating temperature regimes might not be only due to indirect selection against virulence, but also because of decay of unused character due to mutation accumulation (Hall and Colegrave 2008). Our results suggest clearly convergent evolution, and thus, "repeatable" evolution in majority of the traits. There is also a possibility that different starting material could have caused different evolutionary outcome and the evolutionary paths could be more sensitive to the genotypic properties of different ancestors, rather than to the replicate population of the same ancestor (Woods et al. 2011). This brings up an intriguing question about the role of starting material in leading to a particular evolutionary outcome in experimental evolution studies (Travisano et al. 1995), which, however, is beyond the scope of this article.

As a conclusion, our results give strong evidence for the evolution of thermal generalism in response to selection imposed by fluctuating temperature. In addition, we found that general tolerance for several novel environments can evolve as a correlated response to this adaptation (Lee and Gelembiuk 2008). Interestingly, however, adaptation to fluctuations led to attenuated virulence. Given the fact that current climate scenarios predict increases in thermal fluctuations in the future (IPCC 2007), our results suggest that species invasiveness and virulence could potentially change as a consequence of evolution of thermal generalism.

## ACKNOWLEDGMENTS

The authors thank V. Mänttäri, N. Onttonen, J. Ruth, and S. Lahtinen for laboratory assistance, E. Ihalainen for editing the language, C. Nokkala for providing the flies, Academy of Finland (Projects 7128888 [TK], 1130724 and 1255572 [JL]), Finnish Cultural Foundation (LM), and Marie-Curie Intra-European Fellowship within the 7th European Community Framework Programme (VPF) for funding, and Centre of Excellence in Evolutionary Research and Centre of Excellence in Biological Interactions for funding and facilities.

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Associate Editor: C. Lee