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Diversification in temporally heterogeneous environments: effect of the grain in experimental bacterial populations

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Abstract

Although theory established the necessary conditions for diversification in temporally heterogeneous environments, empirical evidence remains controversial. One possible explanation is the difficulty of designing experiments including the relevant range of temporal grains and the appropriate environmental trade-offs. Here, we experimentally explore the impact of the grain on the diversification of the bacterium *Pseudomonas fluorescens* SBW25 in a temporally fluctuating environment by including 20 different pairs of environments and four temporal grains. In general, higher levels of diversity were observed at intermediate temporal grains. This resulted in part from the enhanced capacity of disruptive selection to generate negative genotypic correlations in performance at intermediate grains. However, the evolution of reciprocal specialization was an uncommon outcome. Although the temporal heterogeneity is in theory less powerful than the spatial heterogeneity to generate and maintain the diversity, our results show that diversification under temporal heterogeneity is possible provided appropriate environmental grains.

Introduction

Environmental heterogeneity is a major determinant of species richness within communities (Hutchinson, 1961; MacArthur & Levins, 1964; Levins, 1968; Chesson, 2000) and of genetic polymorphism within species (Levene, 1953; Dempster, 1955; Hedrick, 1986). In contrasted environments, biological diversity can be generated and maintained by divergent selection, resulting in ecological specialization and the concomitant emergence of genotype-by-environment fitness interactions (Felsenstein, 1976; Bell, 1990).

Levins (1968) showed that a stable diversification in heterogeneous environments requires strong evolutionary constraints (trade-offs) on the traits involved in adaptation, such that specialist types perform well in some but poorly in other environments and that generalist types have a lower overall mean performance than the specialists. Diversification also depends on the environmental grain, that is, on the spatial or/and temporal scales of environmental heterogeneity relative to the 'home range' of an individual (Levins, 1968; Kassen, 2002). A fine grain means that an individual encounters more than one environmental condition during its lifetime. This is expected to select for an all-purpose generalist. A coarse grain means that the environment remains constant for the entire individual's lifetime or for several consecutive generations. Under such conditions, a single specialist is likely to evolve. Both strong trade-offs and coarse-grained environments are often necessary conditions for diversification via the evolution of specialization (Levins, 1968; Ravigné *et al.*, 2009).

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In temporally heterogeneous environments, the conditions for protected polymorphism are more restricted than in spatially heterogeneous environments (Dempster, 1955; Haldane & Jayakar, 1963; Gillespie, 1972; Felsenstein, 1976; Chesson & Warner, 1981; Hedrick, 1986; Dean, 2005). Although a spatially heterogeneous environment provides refuges for different specialized types, temporal fluctuations regularly expose all types to different environments, thereby facilitating the fixation of a single type with the highest mean performance across all environmental conditions (Haldane & Jayakar, 1963; Levins, 1968; Nagylaki, 1975; Wilson & Yoshimura, 1994; Bürger & Gimelfarb, 2002). As with spatial heterogeneity, evolutionary outcomes may strongly depend on the grain of the fluctuations (Levins, 1968; Bürger & Gimelfarb, 2002). In very frequently changing environments (fine temporal grain), a generalist or single most productive specialists may be favoured (Kassen, 2002) and consequently diversity will be low. Conversely, if the environment remains constant over many generations (coarse temporal grain), the sequential evolution and fixation of specialists adapted to the contemporary environmental conditions are expected. At intermediate fluctuations (grain) however, there may not be sufficient time for specialists to become fixed in the population, thereby allowing periods of coexistence of different specialists (Nagylaki, 1975). As a general pattern, we would therefore expect maximal diversity at intermediate temporal grains. This is consistent with a theoretical model by Bürger & Gimelfarb (2002), predicting highest levels of genetic variance at intermediate periods of fluctuation.

Experimental manipulation of the temporal grain has produced equivocal results regarding the evolution and maintenance of biological diversity (reviewed in Kassen, 2002). Temporal fluctuations in nutrient or resource supply have been shown to facilitate coexistence in bacterial or phytoplankton model systems when compared to single constant environments (Flöder *et al.*, 2002; Kassen, 2002; Suiter *et al.*, 2003; Decamps-Julien & Gonzalez, 2005; Jiang & Morin, 2007). For example, in an environment cycling between high and low nutrient supplies, intermediate cycle length allowed the coexistence of different strains of the bacterium *Escherichia coli*, whereas finer or coarser grains both led to the predominance of a single strain (Suiter *et al.*, 2003). Similarly, Flöder *et al.* (2002) showed that phytoplankton diversity peaked at intermediate rates of fluctuation between high and low light intensities. Some other studies have however found no obvious impact of temporal grain on the amount of diversity (Riddle *et al.*, 1986; Grover, 1988; Litchman, 1998; Scheiner & Yampolsky, 1998) or on the evolution of specialist/generalist strategies (Reboud & Bell, 1997; Kassen & Bell, 1998; Buckling *et al.*, 2007).

Clearly, the results of such experiments critically depend on the inclusion of a sufficiently wide range of temporal grains and on the presence of contrasting environments that can produce strong trade-offs. However, obtaining *a priori* information on these prerequisites may be difficult or time-consuming. As an alternative, rather than focusing in detail on a well-defined single set of fluctuating environments (i.e. one trade-off), one may use a 'shotgun' approach, replicating a large number of sets of environments over a wide range of temporal grains. This approach may only have a limited resolution for individual combinations of environments, but potentially bundles weak individual signals to reveal general properties of diversification as an average effect across many temporally variable environments.

Here, we used such a 'shotgun' approach to study the impact of temporal heterogeneity on the evolution of genotypic diversity in experimental populations of the bacterium *Pseudomonas fluorescens* SBW25, adapting to pairwise combinations of 40 different carbon substrates (environments) and four different temporal grains. Populations were propagated either continuously on each single environment or were alternated between the two environments at different temporal grains: from 1-day (finest grain) to 8-day intervals (coarsest grain). The experiment was replicated over 20 independent pairs of environments from different chemical families, increasing the chances of including different trade-offs. After ~200 generations of evolution (i.e. 32 days), we assayed within-population genotypic diversity in performance on the two environments and estimated the strength of genotype-by-environment interactions (i.e. *inconsistency*, Bell, 1990; Venail *et al.*, 2008).

Material and methods

Study organism

Pseudomonas fluorescens SBW25 has become a bacterial model system for the study of adaptation and diversification in heterogeneous environments (Buckling *et al.*, 2009), in particular because *P. fluorescens* rapidly specializes when propagated in different environments (MacLean *et al.*, 2004; Barrett *et al.*, 2005; Venail *et al.*, 2008). The present experiment was initiated from a single clone already adapted to laboratory conditions by selection for ~900 generations in a complex environment containing eight carbon sources (Barrett *et al.*, 2005).

Selection environments

In a serial transfer experiment (i.e. batch culture), we allowed replicate bacterial populations to diversify for 32 days (~200 generations) in 20 independent pairs of environments. These pairs consisted of two arbitrarily chosen carbon substrates of Biolog GN2® 96-well microplates (Biolog, Hayward, CA, USA). The list of substrates and their arrangement on the Biolog plate are provided as Data S1.

Temporal grain

The experiment was designed such that all replicate populations spent a total of 16 days (~100 generations) in a given environment. For each pair of environments, we used four frequencies of cyclical temporal fluctuations, with the two environments alternating in 1-, 2-, 4- and 8-day intervals, over the total of 32 days. Thus, the coarseness of the temporal grain ranged from relatively fine (1 day or ~every 6.5 generations) to very coarse (8 days or ~ every 52 generations). However, because the capacity of bacteria to exploit carbon substrates is very variable, the real number of generations spent on each environment may vary among substrates. We established a control treatment for each pair of environments where replicate populations were independently cultured on each single environment for 16 days (~100 generations). This is equivalent to a spatially heterogeneous environment (hereafter *spatial* treatment), with two spatially separated subpopulations evolving independently in a temporally constant environment. We expected a maximum of diversification between subdivided populations in this treatment. We established three replicate populations for each combination of pair of environments and treatment (20 pairs × 5 treatments × 3 replicate populations = 300 populations in total), spread out over 13 Biolog plates.

Selection protocol

Aliquots from a frozen stock (−80 °C) of the initial clone were inoculated into 13 sterile glass vials with 6 mL of M9KB solution (NH_4Cl 0.1 g L^{-1}; Na_2HPO_4 0.6 g L^{-1}; KH_2PO_4 0.3 g L^{-1}; NaCl 0.05 g L^{-1}; glycerol 1 g L^{-1}, proteose peptone #2 2 g L^{-1}). After 24 h of growth under constant orbital shaking (2.5 *g*) at 28 °C, 1 mL of culture from each vial was centrifuged (3 min at 6900*g*), the supernatant was removed and replaced with 1 mL of M9 minimal salts medium (NH_4Cl 1 g L^{-1}; Na_2HPO_4 6 g L^{-1}; KH_2PO_4 3 g L^{-1}; NaCl 0.5 g L^{-1}). Then, 125 μL of this washed solution was diluted into 25 mL of M9 medium and starved for 2 h at 28 °C. For the first inoculation of the 13 Biolog plates, we added 140 μL of starved cells to each well. For this and all subsequent transfers, plates were incubated for 24 h in the dark at 28 °C without shaking. To renew the available nutrients and to alternate between environments, small samples of the bacterial populations were transferred to new Biolog plates in daily intervals. At each transfer, we homogenized the content in each well by gently taking up and releasing 100 μL of volume with a pipette (20 times); then, a pin replicator (Boekel 96 pin/well model #140500) was used to transfer ≈2 μL of culture to the respective substrate on a new Biolog plate (each well on the new plate was previously filled with 140 μL of M9 medium). This batch culture technique ensured nearly constant bacterial growth. After 32 transfers (16 in the *spatial* treatment), 80-μL samples from each replicate population were mixed with 80 μL of a 50 : 50 glycerol/M9 medium and stored at −80 °C.

Performance assay

To obtain individual genotypes for the pure-culture performance assays, we streaked out samples from each evolved bacterial population on KB-agar Petri dishes. Eight randomly picked colonies ('genotypes') per population were individually grown on KB, and 400 μL of overnight cultures was frozen in 50% glycerol at −80 °C. Prior to the assay, genotypes were amplified for 24 h on 96-well plates in 140 μL of M9KB medium at 28 °C, under constant orbital shaking (200 r.p.m). Then, the plates were centrifuged (5 min at 3050*g*) and wells washed by removing the supernatant and adding 140 μL of M9 medium. After 2 h of starvation, we used the pin replicator to inoculate the Biolog plates for assays. Genotypes from fluctuating treatments were tested on the two carbon substrates (say, substrates A and B) on which they had evolved; the genotypes from the corresponding *spatial* treatment (separate evolution on either A or B) were also tested on these two substrates. Each genotype was tested once on each substrate (20 pairs of environments × 5 temporal treatments × 3 replicate populations × 8 genotypes = 2400 tests). The assays were carried out on 4 days, with two of the eight genotypes of a given replicate population tested on each day. After 24 h in the dark at 28 °C, we scored the optical density (absorbance) at 590 nm; this test was performed with a BMG LABTECH multidetection microplate reader FluoStar OPTIMA. Light absorbance measures the capacity of the genotype to exploit the carbon source and is used as a proxy for bacterial performance (MacLean & Bell, 2003). Given the size of the experiment, competition fitness assays were impossible to conduct. To assess the potentially confounding effects due to uncontrolled variation among the four assay dates, we included five replicates of the ancestral clone on each day.

Estimates of genotypic diversity

We used phenotypic diversity as proxy for genotypic diversity. To properly assess the genotypic diversity would have required that each genotype be tested at least twice (Bell, 1990). Our calculation of within-population genotypic diversity was based on the performance of the eight randomly sampled genotypes of a given replicate population on two environments (e.g. substrates A and B from each carbon substrate pair, Fig. 1). Our estimators of genotypic diversity were *inconsistency* and the mean *genotypic variance* (*GV*) in performance. *Inconsistency* indicates variation among genotypes in their ranking of performance on each environment, suggesting their specialization to different

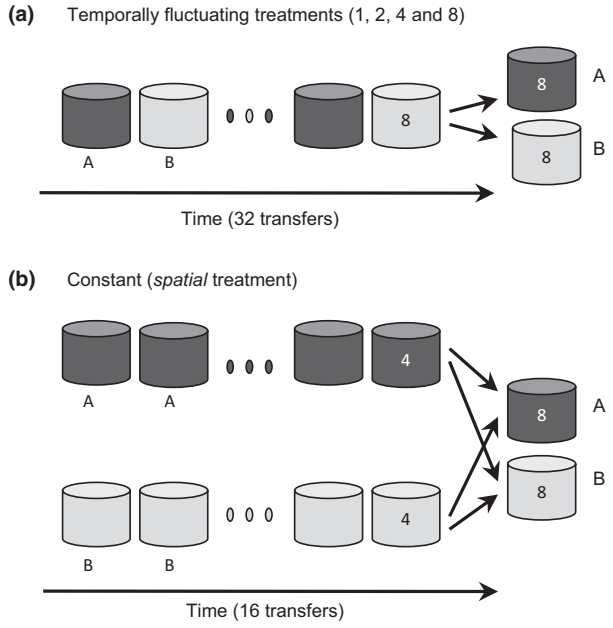

Fig. 1 Flow chart of the experimental design used for calculations of genotypic diversity: *inconsistency and GV*. After 32 transfers, for all the temporally fluctuating treatments (a) (1, 2, 4 and 8) we randomly sampled eight genotypes from each selection line and assayed them on the two sources of the environmental pair (a and b) to calculate the *inconsistency* (eqn 1) and *genotypic variance* (eqn 2). (b) For the spatial treatment, we measured the *inconsistency* and *genotypic variance* by randomly picking four genotypes from each evolved line after 16 days in constant environments and pooling the eight genotypes. This treatment represented a spatially divided population and was used as a theoretical upper boundary for diversification.

conditions (Hall & Colegrave, 2006) and was calculated as:

$$inconsistency = \sigma_{GA}\sigma_{GB}(1 - \rho_{GAGB}) \quad (1)$$

σ_{GA} and σ_{GB} are the standard deviations in performance (i.e. measured light absorbance) among the eight genotypes in environments A and B, and ρ_{GAGB} is the across-genotype correlation of performance between the two environments. *Inconsistency* is zero if the performance rank order of genotypes is perfectly positively correlated among environments (i.e. parallel reaction norms, $\rho_{GAGB} = +1$). Highly positive genotypic correlations indicate the potential for selection of generalist genotypes with high performance in both environments. *Inconsistency* increases as the genotypic correlation coefficient becomes <+1, that is, when there is an increasing number of performance rank order inversions of genotypes between the two environments. Genotypic correlations become negative with substantial rank order inversions (i.e. crossing reaction norms) and as the best performing available genotypes are specialists of different environments. *Inconsistency* is maximal for $\rho_{G1G2} = -1$, when equal number of specialists of the different environments are present.

For each environmental pair and each temporal grain, we also calculated the mean *genotypic variation* (*GV*) in performance as:

$$GV = \frac{\left(\sigma^2_{GA} + \sigma^2_{GB}\right)}{2} \quad (2)$$

where σ^2_{GA} and σ^2_{GB} are the variances in performance among the eight genotypes in environments A and B, respectively. *GV* is an indicator of the differences in genotypic performances. *GV* is high if genotypes perform unequally, suggesting the presence of adapted and maladapted genotypes.

Inconsistency and *GV* are expected to be correlated, as the former increases with the product of the genotypic standard deviations (σ) and the latter with the sum of the variances (σ^2). However, as *Inconsistency* considers rank order inversions via the genotypic correlation coefficient, the two parameters are complementary indicators of diversity.

Inconsistency and *GV* were calculated for each of the three replicate populations from the 20 environmental pairs and five different environmental treatments, including the four *temporal* treatments and the *spatial* treatment. In the *spatial* treatment, genotypes originated from two separate populations propagated on a single constant environment. Thus, for a given pair of constant environments (e.g. substrates A and B, Fig. 1), we randomly chose four of the eight genotypes from environment A and four from environment B to reassemble a virtual group of eight genotypes, for which we calculated *inconsistency* and *GV*. This mimicked the sampling from two isolated subpopulations in a spatially heterogeneous environment.

Response to selection

For each temporal grain and each environment, we quantified the response to selection as the difference in performance between genotypes after 32 days of evolution (16 days for the constant treatment) and the ancestral clone. Thus, we calculated the response to selection in the *contemporary* environment from which genotypes were isolated after the last growth cycle and in the *past* environment that populations had encountered one cycle earlier (for constant treatments the environment they had never encountered). The response to selection in the *contemporary* environment reveals the efficacy of selection in this environment, whereas the response to selection in the *past* environment may help to hint at potential costs of adaptation. The difference in the responses to selection among the two environments (*contemporary* minus *past*) is an indicator of the degree of specific adaptation.

Statistical analysis

Inconsistency was log-transformed and *GV* was square rooted for analysis. We used the factorial analyses of

variance (ANOVA) to test the effects of the temporal grain and the environmental pair on *inconsistency* and *GV*. We performed multiple comparison tests (*t*-tests) to compare *temporal* grains. To test for linear and nonlinear relationships among the temporally alternating treatments, we also carried out analyses of covariance (ANCOVA), with temporal grain as a covariate (first- and second-order polynomial models). If the quadratic term of the second-order polynomial model was significant, we applied a unimodality test (Mitchell-Olds & Shaw, 1987) to determine whether the unimodal relationships reached a maximum of *inconsistency* or *GV* within the range of temporal grains tested. Finally, we fitted linear models to test for a relationship between the mean response to selection and the environmental grain. We used JMP (SAS, 2003) software for statistical analysis.

Results

No block effect on assays

Reference measurements of absorbance of the ancestral strain did not differ among the four experimental blocks ($F_{3,95} = 0.67$, $P = 0.58$), indicating the absence of a 'day effect' on bacterial performance. Therefore, results among evolved lines were analysed without correction for block (i.e. day) effects.

Higher genotypic diversity in the spatial treatment

We found a significant effect of the environmental treatment on *inconsistency and genotypic variation (GV)*, our two estimators of genotypic diversity. Overall, *inconsistency* in the *spatial* treatment was substantially higher than in any of the *temporal* treatments (*spatial* vs. highest *temporal* treatment (4-day interval): $F_{1,95} = 10.17$, $P = 0.0019$; Fig. 2a). Thus, rank order changes of genotypes between environments were more pronounced in the *spatial* treatment than in any of the *temporal* treatments. The *spatial* treatment also produced higher levels of *GV* than did the *temporal* treatments (*spatial* vs. all *temporal* treatments: $F_{1,95} = 11.51$, $P = 0.0010$; Fig. 2b).

Higher genotypic diversity for intermediate temporal grain

Further analysis was restricted to the *temporal* treatments and revealed significant effects of temporal grain and environmental pair on *inconsistency* and *GV* (Table 1 and Fig. 2).

Inconsistency

Generally, environments fluctuating in 1- and 2-day intervals (fine temporal grains) produced lower levels of *inconsistency* than did environments fluctuating in 4- and 8-day intervals (coarse grains; Fig. 2a). The ANCOVA revealed a significant linear (positive) and second-order (negative) effects of temporal grain on *inconsistency* (Table 2). This relationship was largely consistent across environmental pairs, as indicated by the nonsignificant grain × pair interaction (Table 2, Fig. S2). There was a significant unimodal relationship between temporal

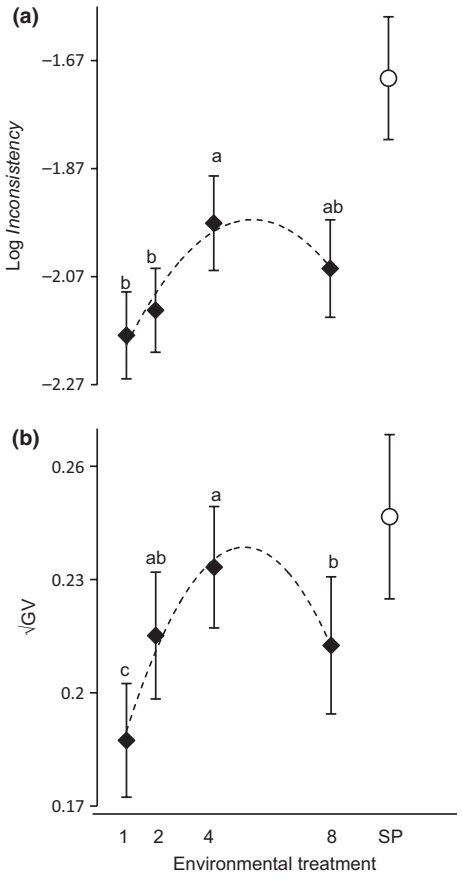

Fig. 2 Effect of temporal environmental grain on genotypic diversity. (a) Higher levels of *inconsistency* were observed when mixing genotypes from two selection lines (*spatial* treatment, open circle) than in any other treatment. Among the temporal treatments, the intermediate grain treatment (4-day) presented higher levels of *inconsistency* than fine grain treatments (1- and 2-day). A slight reduction in *inconsistency* was observed for the 8-day treatment, confirmed by the unimodality test revealing a peak of *inconsistency* within the range of temporal grains included in the experiment (~5.33 days). (b) Higher levels of *genotypic variance* were observed when mixing genotypes from two selection lines (*spatial* treatment, open circle) than in any other treatment. Among the temporal treatments, the 1-day treatment presented the lowest levels of *genotypic variance* and the 4-day treatment the highest. The unimodality test confirmed a peak of *genotypic variance* within the range of temporal grains included in the experiment (~5.04 days). In both panels, each dot represents mean values (±SE) over the 20 environmental pairs replicated each three times. Any two data points that do not share the same letter have significantly different functional diversity ($P < 0.05$, *t*-test). The dashed line represents the unimodal relationship between temporal grain and *inconsistency* or *GV*, respectively.

Table 1 Mixed-model ANOVA of the effect of temporal grain (fixed factor) and environmental pair (random factor) on log *Inconsistency* and *GV*. The effect of grain was tested over the grain × pair interaction, the other terms over the error term. Bold values show statistically significant effects.

Effect	d.f.	M.S.	*F*	*P*-value
Inconsistency				
Grain	3	2.645	3.08	**0.0344**
Pair	19	6.444	7.65	< **0.0001**
Grain × Pair	57	0.858	1.02	0.4542
Error	160	0.843		
GV				
Grain	3	0.0214	8.1	**0.0001**
Pair	19	0.0448	13.24	< **0.0001**
Grain × Pair	57	0.0026	0.78	0.8607
Error	160	0.0034		

Table 2 Mixed-model ANCOVA of the effect of temporal grain (as a covariate) and the environmental pair (random factor) on log *Inconsistency* and square-rooted *genotypic variance* (*GV*). Bold values show statistically significant effects.

Effect	d.f.	M.S.	*F*	*P*-value
Inconsistency				
Grain	1	1.3727	8.95	**0.0032**
Pair	19	0.0557	3.76	< **0.0001**
Grain × Pair	19	0.1975	1.29	0.1958
Grain^2	1	0.8915	5.81	**0.0169**
Grain^2 × Pair	19	0.2249	1.47	0.1024
Error	180	0.1534		
GV				
Grain	1	0.0533	16.26	< **0.0001**
Pair	19	0.0163	4.97	< **0.0001**
Grain × Pair	19	0.0028	0.87	0.6225
Grain^2	1	0.0522	15.92	< **0.0001**
Grain^2 × Pair	19	0.0026	0.80	0.7038
Error	180	0.5905		

grain and *inconsistency* (Fig. 2a, $P < 0.05$), with an estimated peak of *inconsistency* within the range of temporal grains included in the experiment (~5.33 days).

Genotypic variation (GV)

On average, environments fluctuating in a 1-day manner produced the lowest levels of *GV,* whereas the 4-day treatment produced the highest. There was also a significant reduction in *GV* for the 8-day treatment compared to the 4-day treatment (Fig. 2b). The ANCOVA revealed a significant linear (positive) and second-order (negative) effects of temporal grain on *inconsistency* (Table 2). Again, this relationship did not significantly vary among environmental pairs (nonsignificant grain × pair interaction, Table 2, Fig. S3). There was a significant unimodal relationship between temporal grain and *GV* (Fig. 2b, $P < 0.05$), with a peak of *GV* within the range of temporal grains included in the experiment (~5.04 days).

Taken together, our two measures of within-population genotypic diversity revealed quite similar results with maximum values at intermediate temporal grains. Alternating environments in 4-day intervals produced the strongest variation in the relative performance of genotypes between environments (*inconsistency*) and in the mean variation in performance of genotypes within environments (*GV*).

Negative genotypic correlation coefficients were rare

We further inspected in more detail one component of *inconsistency*, the genotypic correlations. Negative genotypic correlations indicate ecological specialization to the different available environments. Overall, negative genotypic correlations were relatively rare (Fig. 3). On average, only 30% (6/20) of the environmental pairs in the *spatial* treatment showed negative genotypic correlations, indicating specialization of genotypes on these environments. Because specialization was more pronounced in the spatially heterogeneous environments, genotypic correlations were on average less positive in the *spatial* treatment (0.28 ± 0.10 SE) than in the temporally variable environments (0.47 ± 0.04 SE). The strength of genotypic correlations did not significantly differ among temporal grains ($F_{4,76} = 0.85$, $P > 0.4$), with only 5% (in the 2-day treatment) to 20% of the pairs (in the 4-day treatment) in the temporal treatments producing negative correlations (Fig. 3). Thus, although analysis of *inconsistency* revealed a clear signal of the temporal grain, patterns of the genotypic correlations indicate that, irrespective of the grain, specialists were relatively rare in the *temporal* treatments.

Responses to selection were positive

On average, responses to selection were positive (0.284 ± 0.006 SE, $n = 200$), indicating considerable adaptation to the environmental conditions. Patterns of responses to selection differed between *contemporary* and *past* environments. First, populations showed stronger adaptation to the substrates they had last encountered in the long-term experiment (*contemporary* environment: 0.448 ± 0.008 SE, $n = 100$) than to the environments they had encountered one cycle back (*past* environment: 0.120 ± 0.008 SE, $n = 100$; $t = -30.404$, $P < 0.0001$). Second, responses to selection on *contemporary* environments linearly increased with coarseness of the environmental grain (main effect of grain: $F_{1,98} = 9.609$; $P = 0.024$), whereas response to selection on *past* environments decreased ($F_{1,98} = 8.7124$; $P = 0.038$; Fig. 4). Thus, the longer the time continuously spent on the last environment they encountered (*contemporary*), the stronger was the degree of adaptation to that environment and

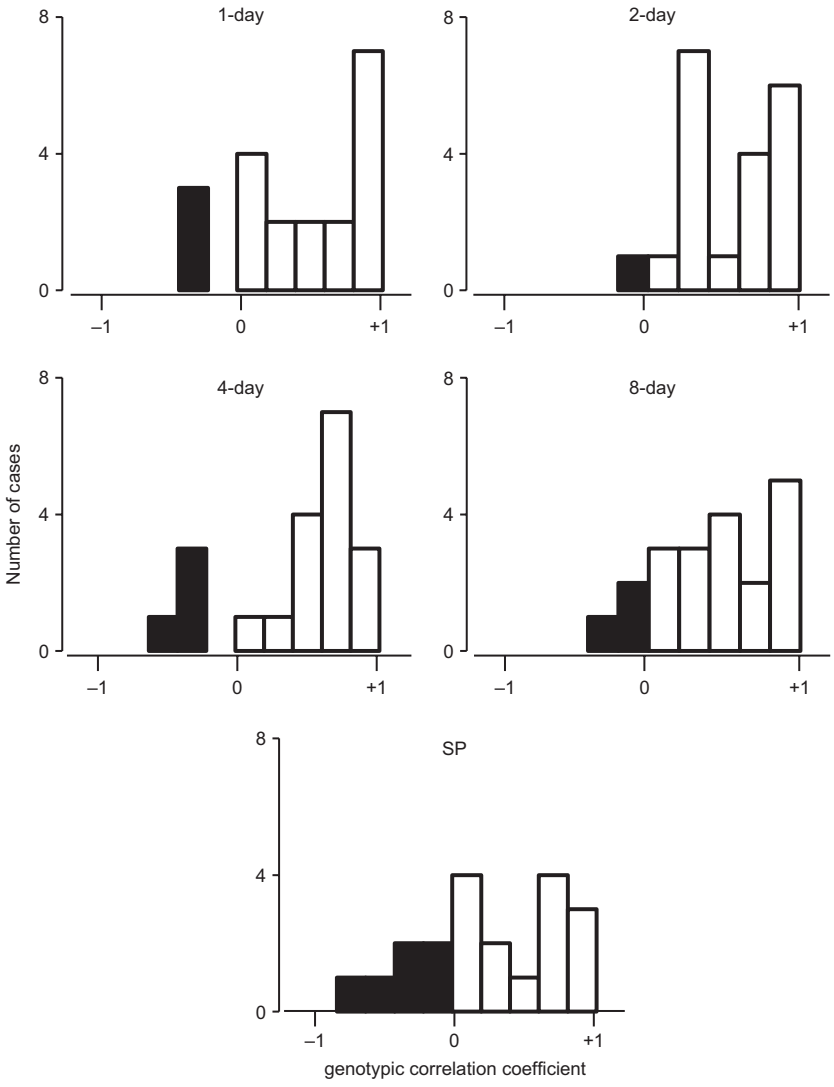

Fig. 3 Frequency distributions of genotypic correlation coefficients for each environmental treatment: SP is for *spatial*. Each distribution represents 20 pairwise comparisons of environmental conditions over eight genotypes. Negative genotypic correlations are indicated in black.

the less they were adapted to the other environment (*past*).

Discussion

Several previous experimental studies investigated the effects of temporal environmental heterogeneity on diversity. In some cases, intermediate fluctuations lead to maximum genotypic or species diversity (Mackay, 1980; Gottschal *et al.*, 1981; Flöder *et al.*, 2002; Suiter *et al.*, 2003), whereas others found no effects of temporal grain on diversity (Scheiner & Yampolsky, 1998; Buckling *et al.*, 2007; Hughes *et al.*, 2007) or a strong dependence on factors such as species identity (Gottschal *et al.*, 1981), environmental gradient intensity (Litchman, 1998) or the phenotypic trait considered (Mackay, 1980). These variable results suggest considerable complexity in the effect of temporal heterogeneity on diversification (Kassen, 2002) and may be explained by the incorporation of a narrow set of experimental conditions (i.e. a single trade-off or a fixed temporal grain). Only the inclusion of a sufficiently wide range of temporal grains and the presence of different trade-offs may produce a general picture of the outcome of diversity on temporally heterogeneous environments. By combining a large number of environmental scenarios along a wide range of temporal grains, our experiment has allowed us to explore the generality of the relationship between temporal heterogeneity and diversity emergence.

Spatial vs. temporal heterogeneity

Consistent with theory (Dempster, 1955; Haldane & Jayakar, 1963; Gillespie, 1972; Felsenstein, 1976; Hedrick, 1986; Dean, 2005), spatial heterogeneity

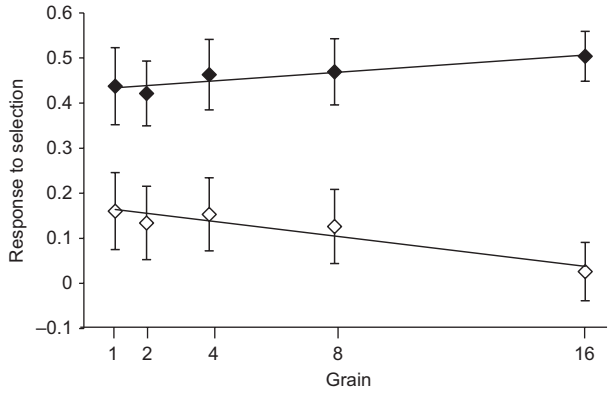

Fig. 4 Effect of temporal grain on the mean response to selection calculated as the difference in performance between the evolved genotypes and the ancestral clone. Filled diamonds represent the response to selection in the last encountered environment (*contemporary* ±SE). Empty diamonds represent the response to selection to the other environment (*past* ±SE). Lines represent linear fits. In the case of the *contemporary* environment, the grain represents the number of continuous days the genotypes have been exposed to that environment. In the case of the *past* environment, the grain represents the number of days the genotypes have not been exposed to that environment.

produced substantially higher levels of diversity than did temporal heterogeneity. The *spatial* treatment, which is equivalent to a 16-day constant treatment, mimicked the sampling from two subpopulations in a spatially coarse-grain heterogeneous environment with each isolated subpopulation being selected in a single constant environment. This coarsest (spatial and temporal) environmental grain should favour the evolution of resource specialists and sets the upper boundary of diversification in our experiment. Indeed, among all treatments, lines from the *spatial* treatment tended to have the strongest mean response to selection on the local contemporary environment and the weakest response to selection on the foreign (not encountered) environment suggesting specialization (Fig. 4). Lines from the *spatial* treatment made little or no significant progress on environments they never encountered. These findings corroborate results from previous studies on *P. fluorescens*, demonstrating the importance of spatial heterogeneity in shaping the emergence and maintenance of diversity (Rainey & Travisano, 1998; MacLean & Bell, 2003; Barrett *et al.*, 2005; Jasmin & Kassen, 2007; Venail *et al.*, 2008).

Effects of temporal grain on genotypic diversity

Fine-grained environmental fluctuations (i.e. 1 and 2-day intervals) produced, in general, lower levels of *inconsistency* and *genotypic variation* than did more coarse-grained fluctuations (i.e. 4 and 8-day). This general pattern held individually for most pairs of substrates tested. Lower diversity is expected in fine grains if frequent switching between environments selects for a generalist adapted to frequent environmental change (with maximum mean performance over both environments) or if a specialist from the most productive environment is taking over the entire population (Kassen, 2002). It is likely that in our experiment the generalists were more frequent in fine than coarse grains treatments as we found that the difference between the mean response to selection in the *contemporary* environment and the mean response to selection in the *past* environment increased with the grain (Fig. 4). With more coarse-grained fluctuations, periods of selection in a given environment may be long enough to select for specialist genotypes adapted to that environment. If selection coefficients are regularly oscillating, specialists adapted to both environments may be maintained (Nagylaki, 1975), thereby increasing the diversity as observed in our experiment at intermediate grains. However, this coexistence of differentially adapted specialists may be limited, as very coarse temporal grains with long periods of growth in a given environment should provide sufficient time for selection to eliminate maladapted genotypes. This would explain why average levels of *GV* and *inconsistency* levelled off or even decreased at the highest temporal grain in our experiment (i.e. 8-days). These observations are consistent with results from a theoretical model by Bürger & Gimelfarb (2002), who showed that additive genetic variance can be maximized at intermediate periods of environmental fluctuations.

Here, we were mainly concerned with overall patterns emerging across many environmental pairs (i.e. trade-off shapes), and the statistically nonsignificant pair-by-grain interactions (Tables 1 and 2) indicates that such general effects indeed exist. Nonetheless, visual inspection of individual pairs (Figs S2 and S3) suggests some degree of variation. For example, flat relationships between grain and diversity may reflect weak trade-offs for certain pairs of substrates, resulting in positive correlated responses to selection and the evolution of generalists, irrespective of the temporal grain. Moreover, even if a trade-off exists, very low initial fitness of the founder population may lead to a fitness increase in both substrates, thus delaying the differentiation of specialists. Clearly, more detailed tests for particular pairs of substrates would require larger sample sizes and/or additional temporal grains, and most importantly, a priori knowledge of trade-off shapes.

Responses to selection

The idea that more coarse-grained temporal fluctuations allow more efficient sorting of adapted genotypes is consistent with the positive relationship between environmental grain and the responses to selection in *contemporary* environments. This positive correlation indicates a link between the time populations had spent on the substrate they had last encountered and the

degree of adaptation observed on this substrate. Furthermore, although the constant (16-day) treatment produced the strongest response to selection in *contemporary* environments, the average response to selection to *past* (or in this case never encountered) environments in this treatment was not significantly different from zero, indicating little or no adaptation to substrates that had never been encountered. This is different from the *temporal* treatments (1- to 8-days), which showed positive responses to selection not only for the *contemporary* environments but also for environments that had been encountered in the past. Such concerted adaptation to multiply experienced substrates has also been observed in other studies with *P. fluorescens* (Barrett *et al.*, 2005; Jasmin & Kassen, 2007). We note, however, that the overall number of generations of evolution differed between the *temporal* (~200 generations) and the constant *spatial* treatment (~100 generations). Thus, positive correlated responses to selection may have arisen in the *spatial* treatment with additional generations of selection (MacLean & Bell, 2002, 2003; MacLean *et al.*, 2004).

Transient and stable polymorphism

Strong specialization of genotypes would have been characterized by negative genotypic correlations. However, in our experiment, genotype × environment interactions, and thus *inconsistency,* arose mainly from imperfect positive genetic correlations ($\rho < +1$), and only a few negative correlations (Fig. 3), similar to the results of a study on a unicellular alga (Bell, 1990). Negative genetic correlations depend on the presence of strong trade-offs and also on the presence of enough genetic variance in the population. A trade-off may exist but not show up because the appropriate genetic variation is lacking or is masked by positive genetic correlations with other traits. Hence, our finding of higher levels of *inconsistency* at coarser temporal grains suggests that the ranking of genotypes varied to some degree between environments, but that complete rank reversals were by far not the rule. In other words, these populations seem to harbour a mix of moderately specialized and generalist genotypes. Unless stronger trade-offs and negative correlations arise after longer selection on these substrates (Buckling *et al.*, 2007), it would appear that some of the observed diversity is only transient and that generalists could become fixed in the long run.

The question of transient vs. stable polymorphism has a long tradition in ecological models of species coexistence in fluctuating environments. The 'Intermediate Disturbance Hypothesis' predicts maximal species diversity at intermediate rates and/or intensities of perturbations (Hutchinson, 1961). Although not stable in the long-term (Chesson & Huntly, 1997), exclusion likely occurs slowly and thus transient diversity may last almost indefinitely, given the appropriate environmental fluctuations (Huston, 1979). Clearly, our adaptation assay only represents a single snapshot in time, and therefore it is difficult to make predictions about long-term stability of the observed diversity. However, our founder populations were genetically uniform, suggesting that the within-population diversity after 200 generations of evolution in temporally heterogeneous environments reflects a balance between mutation and selection. As such, this is an important finding, given that conditions for the emergence of genetic polymorphism may be even more restricted than for its maintenance, as shown at least for spatial variation (Ravigné *et al.*, 2009).

Conclusions

Our results suggest the evolution of diversity in temporally heterogeneous environments follows more complex dynamics than assumed by simple equilibrium theory based on reciprocal specialization (Levins, 1968; Kassen, 2002). The emergence and maintenance of genotypic variation in our experiment likely reflected cases of stable or transient coexistence of specialists and generalists, as well as cases of exclusion by generalists and/or specialists. This calls for more integrated modelling approaches combining evolutionary and ecological processes as well as equilibrium and transient dynamics into a single framework (Vellend & Geber, 2005). Despite this complexity, our 'shotgun' design revealed a general relationship between temporal grain and the amount of biological diversity that emerged through evolution. This suggests that populations, although experiencing different environments, may evolve along similar evolutionary trajectories, because selection follows the same general rules under the same type of fluctuation. Furthermore, our analysis indicates the evolution of a certain level of specialization and thus some of the observed polymorphism may indeed be stably maintained as long as environmental fluctuations occur.

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References

Barrett, R.D.H., MacLean, R.C. & Bell, G. 2005. Experimental evolution of *Pseudomonas fluorescens* in simple and complex environments. *Am. Nat.* **160**: 470–480.

Bell, G. 1990. The ecology and genetics of fitness in *Chlamydomonas*. I. Genotype-by-environment interaction among pure strains. *Proc. R. Soc. Lond. Ser. B.* **295**: 295–321.

Buckling, A., Brockhurst, M.A., Travisano, M. & Rainey, P.B. 2007. Experimental adaptation to high and low quality environments under different scales of temporal variation. *J. Evol. Biol.* **20**: 296–300.

Buckling, A., MacLean, R.C., Brockhurst, M.A. & Colegrave, N. 2009. The beagle in a bottle. *Nature* **457**: 824–829.

Bürger, R. & Gimelfarb, A. 2002. Fluctuating environments and the role of mutation in maintaining quantitative genetic variation. *Genet. Res. Camb.* **80**: 31–46.

Chesson, P.L. 2000. Mechanisms of maintenance of species diversity. *Ann. Rev. Ecol. Evol. Syst.* **31**: 343–366.

Chesson, P.L. & Huntly, N. 1997. The role of harsh and fluctuating conditions in the dynamics of ecological communities. *Am. Nat.* **150**: 519–553.

Chesson, P.L. & Warner, R.W. 1981. Environmental variability promotes coexistence in lottery competitive systems. *Am. Nat.* **117**: 923–943.

Dean, A.M. 2005. Protecting haploid polymorphisms in temporally variable environments. *Genetics* **169**: 1147–1156.

Decamps-Julien, B. & Gonzalez, A. 2005. Stable coexistence in a fluctuating environment: an experimental demonstration. *Ecology* **86**: 2815–2824.

Dempster, E.R. 1955. Maintenance of genetic heterogeneity. *C. S. H. Symp. Quant. Biol.* **20**: 25–32.

Felsenstein, J. 1976. The theoretical population genetics of variable selection and migration. *Ann. Rev. Genet.* **10**: 253–280.

Flöder, S., Urabe, J. & Kawabata, Z.-i. 2002. The influence of fluctuating light intensities on species composition and diversity of natural phytoplankton communities. *Oecologia* **133**: 395–401.

Gillespie, J.H. 1972. The effects of stochastic environments on allele frequencies in natural populations. *Theor. Popul. Biol.* **3**: 241–248.

Gottschal, J.C., Nanninga, H.J. & Kuenen, J.G. 1981. Growth of *Thiobacillus* A2 under alternating growth conditions in the chemostat. *J. Gen. Microbiol.* **126**: 85–96.

Grover, J.P. 1988. Dynamics of competition in a variable environment: experiments with two diatom species. *Ecology* **69**: 408–417.

Haldane, J.B.S. & Jayakar, S.D. 1963. Polymorphism due to selection of varying direction. *J. Genet.* **58**: 318–323.

Hall, A.R. & Colegrave, N. 2006. How does resource supply affect evolutionary diversification? *Proc. R. Soc. Lond. Ser. B.* **274**: 73–78.

Hedrick, P. 1986. Genetic polymorphism in heterogeneous environments: a decade later. *Annu. Rev. Ecol. Syst.* **17**: 535–566.

Hughes, B.S., Cullum, A.J. & Bennett, A.F. 2007. An experimental evolutionary study on adaptation to temporally fluctuating pH in *Escherichia coli*. *Physiol. Biochem. Zool.* **80**: 406–421.

Huston, M. 1979. A general hypothesis of species diversity. *Am. Nat.* **113**: 81–101.

Hutchinson, G.E. 1961. The paradox of the plankton. *Am. Nat.* **95**: 137–145.

Jasmin, J.-N. & Kassen, R. 2007. On the experimental evolution of specialization and diversity in heterogeneous environments. *Ecol. Lett.* **10**: 272–281.

Jiang, L. & Morin, P.J. 2007. Temperature fluctuation facilitates coexistence of competing species in experimental microbial communities. *J. Anim. Ecol.* **76**: 660–668.

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

Kassen, R. & Bell, G. 1998. Experimental evolution in *Chlamydomonas*. IV. Selection in environments that vary through time at different scales. *Heredity* **80**: 732–741.

Levene, H. 1953. Genetic equilibrium when more than one ecological niche is available. *Am. Nat.* **87**: 331–333.

Levins, R. 1968. *Evolution in Changing Environments: Some Theoretical Explorations*. Princeton University Press, Princeton, NJ.

Litchman, E. 1998. Population and community responses of phytoplankton to fluctuating light. *Oecologia* **117**: 247–257.

MacArthur, R. & Levins, R. 1964. Competition, habitat selection and character displacement in a patchy environment. *Proc. Natl Acad. Sci. USA* **51**: 1207–1210.

Mackay, T.F.C. 1980. Genetic variance, fitness, and homeostasis in varying environments: an experimental check of the theory. *Evolution* **34**: 1219–1222.

MacLean, R.C. & Bell, G. 2002. Experimental adaptive radiation in *Pseudomonas*. *Am. Nat.* **160**: 569–581.

MacLean, R.C. & Bell, G. 2003. Divergent evolution during an experimental adaptive radiation. *Proc. R. Soc. Lond. Ser. B* **270**: 1645–1650.

MacLean, R.C., Bell, G. & Rainey, P.B. 2004. The evolution of a pleiotropic fitness trade-off in *Pseudomonas fluorescens*. *Proc. Natl Acad. Sci. USA* **101**: 8072–8077.

Mitchell-Olds, T. & Shaw, R.G. 1987. Regression analysis of natural selection: statistical influence and biological interpretation. *Evolution* **41**: 1149–1161.

Nagylaki, T. 1975. Polymorphisms in cyclically-varying environments. *Heredity* **35**: 67–74.

Rainey, P.B. & Travisano, M. 1998. Adaptive radiation in a heterogeneous environment. *Nature* **394**: 69–72.

Ravigné, V., Dieckmann, U. & Olivieri, I. 2009. Live where you thrive: joint evolution of habitat choice and local adaptation facilitates specialization and promotes diversity. *Am. Nat.* **174**: E141–E169.

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

Riddle, R.A., Dawson, P.S. & Zirkle, D.F. 1986. An experimental test of the relationship between genetic variation and environmental variation in *Tribolium* four beetles. *Genetics* **113**: 391–404.

SAS 2003. *JMP Statistics and Graphics Guide (Version 5.0.1.2)*. SAS Institute, Cary, NC.

Scheiner, S.M. & Yampolsky, L.U. 1998. The evolution of *Daphnia pulex* in a temporally varying environment. *Genet. Res.* **72**: 25–37.

Suiter, A.M., Banziger, O. & Dean, A.M. 2003. Fitness consequences of a regulatory polymorphism in a seasonal environment. *Proc. Natl Acad. Sci. USA* **100**: 12782–12786.

Vellend, M. & Geber, M.A. 2005. Connections between species diversity and genetic diversity. *Ecol. Lett.* **8**: 767–781.

Venail, P.A., MacLean, R.C., Bouvier, T., Brockhurst, M.A., Hochberg, M.E. & Mouquet, N. 2008. Diversity and productivity peak at intermediate dispersal rate in evolving metacommunities. *Nature* **452**: 210–214.

Wilson, D.S. & Yoshimura, J. 1994. On the coexistence of generalists and specialists. *Am. Nat.* **144**: 692–707.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 Arrangement of the 20 pairs of environments on the 96-well Biolog plate

Figure S1 List of environmental pairs

Figure S2 Effect of environmental treatment on *inconsistency* for each environmental pair (P1–P20)

Figure S3 Effect of environmental treatment on *genotypic variance* for each environmental pair (P1–P20)

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