Maintenance of Polymorphism in Spatially Heterogeneous Environments: An Experimental Test of Hard and Soft Selection

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***Abstract*—Predicting and managing contemporary adaption requires a proper understanding of the determinants of genetic variation. Spatial heterogeneity of the environment may stably maintain polymorphism when habitat contribution to the next generation can be considered independent of the degree of adaptation of local populations within habitats (*i.e.*, under soft selection). In contrast, when habitats contribute proportionally to the mean fitness of the populations they host (hard selection), polymorphism is not expected to be maintained by selection. Although mathematically established decades ago, this prediction had never been properly tested. Here we provide an experimental test in which polymorphic populations of *Escherichia coli* growing in heterogeneous habitats were exposed to hard and soft selection regimes. As predicted by theory, polymorphism was longer preserved in asymmetric environments under soft selection. Complementary tests established that soft selection actually protected polymorphism even when one genotype was present at low frequency.**

INTRODUCTION

Genetic variation is the fuel of evolution. Understanding the ultimate forces that shape the amount of genetic variation within populations is therefore a central issue of evolutionary biology. Beyond its fundamental interest, this topic is also crucial for a number of applied issues where evolutionary potential matters. In conservation biology, for instance, preserving the adaptive potential of endangered species is now a primary goal of management policies (Crandall *et al.* 2000). Similarly, as pathogen evolution regularly ruins management attempts (*e.g.*, antibiotic resistance, plant resistance breakdown), managing pathogen polymorphism is becoming a growing concern (Vale 2013).

The spatial heterogeneity in selection pressures among the different habitats that compose an environment constitutes a good explanation of the huge amount of genetic variation observed *in natura*. Yet theoretical works have previously shown that spatial heterogeneity does not necessarily lead to the stable maintenance of local adaptation polymorphism (Dempster 1955, Christiansen 1974, de Meeûs *et al.* 1993, see Kassen 2002, Ravigné *et al.* 2009, Massol 2013, Vale 2013 for reviews). Whether selection leads to the stable maintenance of diversity depends on the interaction between several factors; the existence and strength of local adaptation trade-offs (*i.e.* negative genetic correlations in fitness across different habitats, Levins 1962), the frequency and productivity of the different habitats in the environment (Levene 1953), and the amount of gene flow between habitats (Maynard Smith 1966, Christiansen 1975, Débarre and Gandon 2011). Moreover, very early models showed that the possibility for stable polymorphism crucially depended on local populations within the different habitats, and thus habitats themselves contribute to the next generation (Levene 1953, Dempster 1955, Christiansen 1975, Maynard Smith and Hoekstra 1980). In some organisms, the contribution of local populations to the next generation is fairly independent of their genetic composition. Density regulation limits the productivity of local populations so that better

adaptation to their habitat does not translate into higher local productivity. This can be observed in solitary insect parasitoids that can only lay one egg per host individual (Mackauer 1990). In pathogens, host death also constitutes a mechanism limiting transmission between hosts due to decreases in host accumulation. In this case, pathogens infecting the same host are a local population, so that the density regulation of the contribution of the population is suspected to be the rule rather than the exception (de Meeûs *et al.* 1998, Chao *et al.* 2000). This type of density regulation produces a selection regime called soft selection (Levene 1953, Wallace 1975) that has theoretically been shown to be prone to diversification and polymorphism maintenance.

In contrast, in other species and environments under hard selection, habitat contribution to the next generation is not a fixed characteristic but rather depends on the genetic composition of the local population, *i.e.*, better adaptation implies greater habitat contribution to the next generation (Dempster 1955, Wallace 1975). Hard selection, in principle, hampers diversification and polymorphism maintenance and is expected when population density is not regulated locally within each population but globally at the scale of the environment. It can also be observed in cases where adaptation increases the carrying capacity of the habitat through, *e.g.*, a more efficient use of nutrients. In the case of pathogens for instance, this type of selection occurs when transmission does not depend on whether the host is dead or alive. Hence hard selection is likely frequent in serial passage experiments when parasite transmission is simulated by experimenters. Logically, most serial passage experiments lead to a decrease or disappearance of the initially present polymorphism (for review, see Ebert 1998).

Despite a vast consensus among theoreticians over the importance of the selection regime for polymorphism maintenance in heterogeneous environments, the concepts of hard and soft selection generally remain overlooked in the empirical literature. Hard and soft selections have recently become an explicit concern in studies measuring selection strength and mutation accumulation (Juenger *et al.* 2000, Kelley *et al.* 2005, Laffafian *et al.* 2010, Wade *et al.* 2010, Johnson *et al.* 2011). Despite their relevance, the terms hard and soft selection are still not mentioned in many fields where considering selection regime could be important for analyses. For example, these concepts could be particularly useful for understanding plant pest evolution in landscapes composed of mixtures of plant varieties or the evolution and management of antibiotic resistance.

It must be recognized that despite several important attempts (*e.g.*, Bell 1997), proof of concept − through a proper experimental test − has yet to be made (Vale 2013). Some experiments did test the effect of spatial heterogeneity on genetic variability (reviewed in Rainey *et al.* 2000, Kassen 2002, see also Jasmin and Kassen 2007), most of them concluding that populations confronted with a spatially heterogeneous environment are more variable than those exposed to homogeneous environments. Yet, these experiments did not control for the selection regime imposed by serial passages and experimentally applied hard selection (but Garcìa-Dorado *et al.* 1991, Bell and Reboud 1997). The higher variability observed under these heterogeneous treatments admittedly lies in transient polymorphism being less efficiently removed from heterogeneous environments than from homogeneous environments (see Bell and Reboud 1997 in which unexpected selection was suspected to have played a role). One study explicitly imposed hard and soft selection regimes on a mixture of strains of the unicellular algae *Chlamydomonas reinhardtii* maintained in a heterogeneous environment for 50 generations without sexual reproduction (Bell 1997). Contrary to theoretical predictions, genetic variation remained similar regardless of the type of density regulation. This unexpected result was interpreted as a consequence of the specific nature of the environmental heterogeneity – habitats were composed of different mixtures of nutrients – that did not impose a trade-off in local adaptation (Bell 1997). In the absence of such trade-offs, even under soft selection, polymorphism is not selected for.

Here we aimed at experimentally testing the hypothesis that soft selection can produce the negative frequency-dependence required for stable maintenance of polymorphism while hard selection cannot (Karlin and Campbell 1981). To create the local adaptation trade-off required for polymorphism maintenance, polymorphic *Escherichia coli* populations were built using two genotypes, one being resistant to tetracycline and the other to nalidixic acid. These populations were grown in three heterogeneous environments each composed of two different habitats, one containing a very low concentration of tetracycline and the other a very low concentration of nalidixic acid. Low antibiotic concentrations provided a selective advantage to the resistant genotype over the susceptible one, but both genotypes could grow in all conditions. Three different trade-offs were produced by varying habitat productivities. As in Bell (1997), serial passages were controlled to apply either hard selection (*i.e.*, by transferring an aliquot of each environment) or soft

selection (*i.e.*, by transferring a fixed number of cells from each environment). The duration of the experiments was kept short enough to avoid the emergence (by *de novo* mutation) of a generalist genotype. Whether observed polymorphisms resulted from negative-frequency-dependent selection or not was checked *a posteriori*, through a complementary experiment with different initial genotype frequencies. The evolution of genotype frequencies over the course of the experiment was precisely monitored and systematically compared to theoretical predictions obtained assuming either hard or soft selection.

MATERIALS AND METHODS

Bacterial strains

The *E. coli* B strains used in this study, REL4548 YFP-Tet^R and REL4548 CFP-Nal^R derive from the strain REL4548 kindly provided by R. E. Lenski. REL4548 was evolved in Davis minimal (DM) medium supplemented with 25 µg/mL glucose (DM25) for 10,000 generations as part of a long-term evolution experiment (Lenski *et al.* 1991). Gallet *et al.* (2012) then inserted YFP and CFP genes at the *rhaA* locus of REL4548 using a technique developed by Datsenko and Wanner (2000). A mini-Tn*10* derivative 104 — which contains a tetracycline resistance cassette (Kleckner *et al.* 1991) — was introduced at the *insL-1* locus into REL4548 YFP (clone T121) (Gallet *et al.* 2012) to construct REL4548 YFP-Tet^R. The strain REL4548 CFP-Nal^R was then created by selecting a resistant REL4548 CFP colony on a LB plate (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract; 15 g agar, 1000 mL H_2O) supplemented with 20 µg/mL of nalidixic Acid. These constructions permitted the association of a specific antibiotic resistance with a specific fluorescent marker and therefore easily identifying resistant strains by their fluorescence. Bacterial strains were stored at -80°C in 15 % glycerol stocks.

Habitats

Four habitats (*i.e.*, growth media) were used. Each habitat hosted a single local population. They differed in productivity (*i.e.*, glucose concentration in growth medium), and/or by the presence of very low concentrations of either tetracycline or nalidixic acid. All media were made on the base of Davis minimal (DM) medium (KH_2PO_4 monohydrate 5.34 g/L, KH_2PO_4 2 g/L, ammonium sulfate 1 g/L, sodium citrate 0.5 g/L). Bottles were weighted before and after autoclaving and sterile milliQ water was added to compensate for evaporation happening during sterilization. After autoclaving, media were supplemented with 806 µL/L of $MgSO_4^{2-}$ [1 M], 1 mL/L Thiamine (vitamin B1) [0.2%]. Then, 40 µL/L or 1 mL/L of glucose [2.5%], were added in order to make DM2 and DM50 (2 and 50 µg/mL of glucose being present in the medium, respectively). These media were equivalent to the one used by Lenski *et al.* (1991), but with different glucose concentrations. Tetracycline and nalidixic acid were added at final concentrations of 0.02 µg/mL and 0.7 µg/mL respectively for the first experiment and 0.03 µg/mL and 0.8 µg/mL respectively for the second experiment. Habitats were hereafter denoted Nal2, Nal50, Tet2, and Tet50 depending on the antibiotic used and their productivity as measured through DM concentration.

Environments

Three different environments were used, each composed of two habitats. These three different environments correspond to three different local adaptation trade-offs. In one environment, habitat productivities were comparable (environment B in figure 1B, composed of Nal50 and Tet50 habitats, hereafter referred to as ‘symmetric’ environment). In the two other environments (hereafter ‘asymmetric’ environments), one habitat was more productive than the other (environment A on figure 1B was composed of Nal2 and Tet50 habitats and environment C was composed of Nal50 and Tet2 habitats). While in environment B, both bacterial genotypes had similar mean fitness in the habitat they were adapted to, environments A and C led to favor one genotype over the other at the scale of the whole environment (Figure 1B).

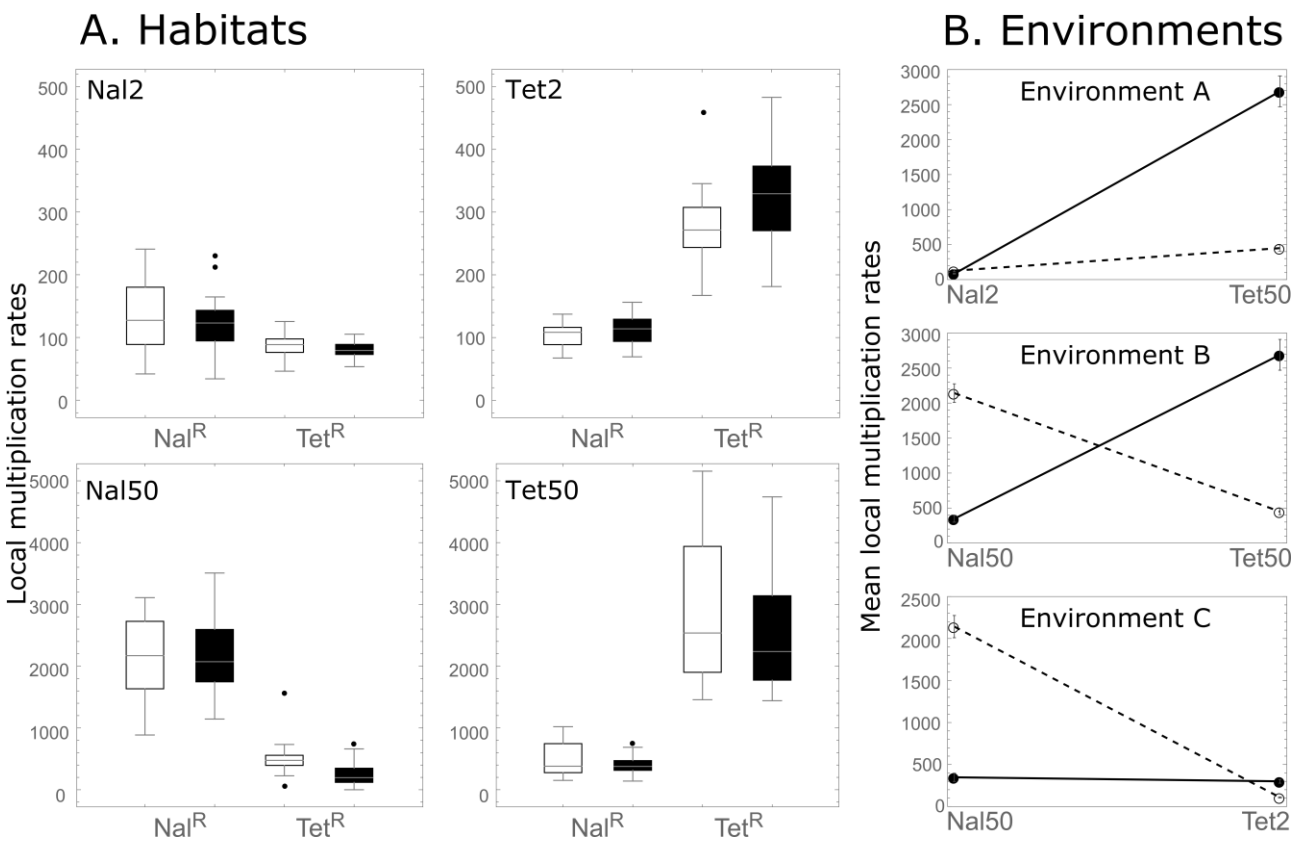

Figure 1: Habitats, environments and local adaptation trade-offs. Local fitness is defined as the rate of multiplication within one habitat between transfers (thereby corresponding to viabilities in Levene's model). Panels: A. Distributions of the local multiplication rates of Nal^R and Tet^R genotypes in the long (white whisker charts) and the short (black whisker charts) trials of Experiment 1 for the four habitats used. B. Mean local multiplication rates of Nal^R (open circles and dashed line) and Tet^R (filled circles and plain line) genotypes in the three environments used (with confidence intervals over all replicates and transfers of Experiment 1).

Experiment 1: Maintenance of established polymorphism

In a first experiment (hereafter referred to as Experiment 1, figure 2), populations with initially equal frequencies of both genotypes were grown under hard selection and soft selection regimes. For each of these two selection regimes, three replicate populations were used for each of the three environments (3 replicates × 3 environments × 2 selection regimes = 18 populations in total). Before the start of the experiment, REL4548 YFP-Tet^R and REL4548 CFP-Nal^R genotypes were grown separately overnight in 5 mL of DM25 (37°C, 215 rpm). At T0, the optical density (OD, 600nm, Eppendorf spectrophotometer) of each culture was measured and a 50/50 mix was made to inoculate all habitats of all environments. At the end of each day (Figure 2), a starting bacterial population was prepared by mixing the bacterial populations from the two habitats. Depending on the selection regime, either the same volume (50 µL − hard selection) or different volumes (containing 10^7 cells per habitat − soft selection) where added to the mix, with a 10-fold dilution in DM0 (*i.e.*, DM medium containing no glucose). Part of the mix was used to make a glycerol stock (stored at -80°C) for subsequent flow cytometer analysis while the other part was used to inoculate both habitats of the environment of the next passage (50 µL into 5 mL of fresh media − an additional 100-fold dilution). Populations were grown overnight (37°C, 215 rpm, 18h of incubation). The whole experiment was replicated twice. In the first trial, flow cytometer measurement showed that the realized initial frequency of REL4548 YFP-Tet^R was 0.508 and the experiment was conducted over five transfers. The second trial, conducted simultaneously with Experiment 2, started from an initial frequency of REL4548 YFP-Tet^R of 0.437 and lasted for three transfers.

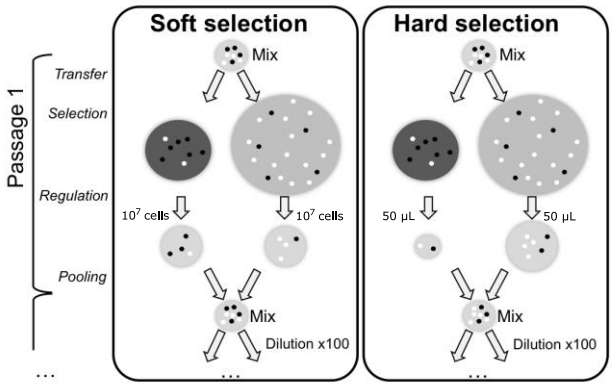

Figure 2. Experimental design. Light and dark grey circles represent habitats supplemented with tetracycline and nalidixic acid respectively. Circle sizes illustrate the nutrient richness of these habitats (large circles = DM50, small circles = DM2). This figure illustrates environment C (Tet2-Nal50). "Mix" grey circles represent mixing tubes. Tiny black and white circles represent Tet^R and Nal^R cells respectively. Black cells have a selective advantage in the dark grey environment, while white cells have a selective advantage in the light grey environment. Populations were transferred daily for 5 transfers. The experiment starts with the transfer of a 50/50 Nal^R - Tet^R mix in each habitat. During the selection step of the experiment, cells grow in the habitat. The amount of cells transferred during the regulation step depends on the selection treatment. Under hard selection fixed volumes (50 µL) of each habitat were pooled together in the mixing tube, while under soft selection fixed numbers of cells (10^7 cells) from each habitat were pooled.

Experiment 2: Polymorphism protection

To go further and test the hypothesis that soft selection can maintain genetic diversity indefinitely by producing negative frequency-dependence, while hard selection cannot, we conducted a complementary experiment (Experiment 2) testing for polymorphism protection. Polymorphism is protected by negative frequency-dependent selection if and only if both genotypes increase in frequency when rare (Prout 1968). We therefore applied hard and soft selection on initial populations where the genotype with a global disadvantage in the considered environment was rare. In Environment A (Nal2-Tet50) the initial frequency of REL4548 YFP-Tet^R was 0.975. In Environment C (Nal50-Tet2) the initial frequency of REL4548 YFP-Tet^R was 0.035. In Environment B (Nal50-Tet50), initially conceived as symmetric, both initial frequencies were tested. The experiment was conducted over two transfers only.

Flow cytometry

Flow cytometry was performed on a Gallios flow cytometer (Beckman Coulter Inc) designed to detect small objects such as bacteria. We used flow cytometry to estimate (*i*) the relative genotype frequencies and (*ii*) cell concentration. This procedure was performed on overnight cultures and on mixes. To estimate cell concentration, fluorescent beads of known concentrations (AccuCount Fluorescent Particles, 7.0-7.9 µm, Spherotech) were added to the cells. Results were analyzed with the Kaluza 1.3 software (Beckman coulter Inc).

Local fitness measurements

The local fitnesses of the two bacterial genotypes in the four habitats were measured in two complementary manners. Firstly, using flow cytometry, for each habitat, the rate of multiplication of each genotype between two transfers was computed as the ratio of cell concentration at the end of the overnight culture over cell concentration at the beginning. These multiplication rates are akin to viability coefficients as defined in hard selection models and could therefore be directly used to feed Levene's and Dempster's equations to establish theoretical predictions. Secondly, for the sake of comparison with other works on bacteria, selection coefficients (*sensu* Chevin 2011) were calculated:

$$s = r_Y - r_C \quad (1)$$

with

$$r_X = \ln \frac{K_{X,f}}{K_{X,i}} \quad (2)$$

where $K_{X,i}$ and $K_{X,f}$ are respectively the initial and final effective of genotype X. Selection coefficients, available in Appendix S1, were used to confirm that no evolution towards a generalist phenotype was observed during the experiment.

Theoretical predictions

Given the viability coefficients $W_{i,j}$ of genotype i in habitat j, under soft selection, the change in frequency p_t of Tet^R bacteria from transfer t to transfer $t + 1$ is governed by the following equation:

$$p_{t+1} = \frac{p_t}{2}\left(\frac{W_{Tet^R,Nal}}{p_t W_{Tet^R,Nal}+(1-p_t)W_{Nal^R,Nal}} + \frac{W_{Tet^R,Tet}}{p_t W_{Tet^R,Tet}+(1-p_t)W_{Nal^R,Tet}}\right). \quad (3)$$

Under hard selection, the trajectory of the frequency p_t of Tet^R bacteria is given by:

$$p_{t+1} = \frac{p_t\left(W_{Tet^R,Nal}+W_{Tet^R,Tet}\right)}{p_t\left(W_{Tet^R,Nal}+W_{Tet^R,Tet}\right)+(1-p_t)\left(W_{Nal^R,Nal}+W_{Nal^R,Tet}\right)} \quad (4)$$

In principle it is thus possible to compare the observed trajectories of genotype frequencies to theoretically expected ones under both selection regimes. Local fitnesses imposed by habitats to the two genotypes were experimentally variable (Figure 1A). To account for such experimental variability, 10,000 trajectories of Tet^R frequency over transfers were simulated by random sampling, at each transfer, of the values of viability coefficients $W_{Tet^R,Nal}$, $W_{Tet^R,Tet}$, $W_{Nal^R,Nal}$, and $W_{Nal^R,Tet}$ among all corresponding values observed over all transfers of Experiment 1 for each habitat. The median and 2.5th and 97.5th percentile values of the distribution of Tet^R frequency at each transfer were used to represent the theoretically expected trajectories of genotype frequency. Similarly, the equilibrium Tet^R frequency was estimated using the median, 2.5th and 97.5th percentile values of the distribution of predicted Tet^R frequencies after 100 transfers.

RESULTS

Three heterogeneous environments with clear local adaptation trade-off

Figure 1A shows the local fitnesses (*i.e.*, between transfer multiplication rates) obtained for each bacterial genotype over all replicates in each of the four habitats. From this and the computation of selective coefficients (available in appendix S1), the existence of three different local adaptation trade-offs could be verified (Figure 1B). It was also confirmed that local fitnesses were similar in the two independent trials of Experiment 1 and that multiplication rates – hence bacteria – did not evolve during the experiment. Theoretical predictions showed that Environment A (Nal2-Tet50) was so asymmetric that the fixation of the Tet^R genotype was expected under both hard and soft selection (Figure 3A and B, right hand side of the x-axis). The expected dynamics of genotype frequency however differed clearly between hard and soft selection (grey areas in figure 3A and B). In Environments B (Nal50-Tet50) and C (Nal50-Tet2), soft selection was expected to lead to polymorphism maintenance (Figure 3D and F), while hard selection was expected to lead to the fixation of one of the two genotypes (Tet^R in Environment B and Nal^R in Environment C, figure 3C and E). In Environment B, the dynamics of genotype frequencies over 5 transfers were hardly distinguishable between hard and soft selection (Figure 3C and D).

Effects of selection regimes on the maintenance of polymorphism

Under hard selection, in both environments with asymmetric habitat productivities (Environments A − Nal2-Tet50 and Environment C − Nal50-Tet2), polymorphism was almost completely lost over the experiment (Figures 3A and E). In environment A, Tet^R genotype frequency reached an average of 0.991 ± 0.001 after 3 transfers in the two Experiment 1 trials (n=6 replicates) and 0.999 ± 0.0002 after 5 transfers in the long trial (n=3). In environment C, Tet^R genotype frequency decreased to 0.032 ± 0.027 after 3 transfers in the two Experiment 1 trials (n=6) and 0.002 ± 0.003 after 5 transfers in the long trial (n=3). The trajectories of genotype frequencies fit well with predictions obtained assuming hard selection (dark grey in figures 3A and E) and fell outside the 95% envelop of theoretical predictions obtained for soft selection (dotted lines in figures 3A and E). In the symmetric environment (Environment B), as predicted, polymorphism was almost unchanged at the end of the experiment with only a slight increase of Tet^R frequency. On average, Tet^R genotype frequency reached 0.595 ± 0.055 after 3 transfers in the two trials of Experiment 1 (n=6 replicates) and 0.593 ± 0.059 after 5 transfers in the long trial (n=3).

Under soft selection, genetic polymorphism was maintained throughout the experiment regardless of habitat productivities (Figures 3B, 3D and 3F). In Environment A (Nal2-Tet50), the frequency of Tet^R bacteria increased at a rate compatible with predictions obtained under soft selection (light grey area in figure 3B) and not with predictions obtained under hard selection (dotted lines in Figure 3B). In this environment, although the expected final outcome of selection was the same under hard and soft selection regimes (fixation of Tet^R bacteria), the rate of evolution was much slower under soft selection than under hard selection.

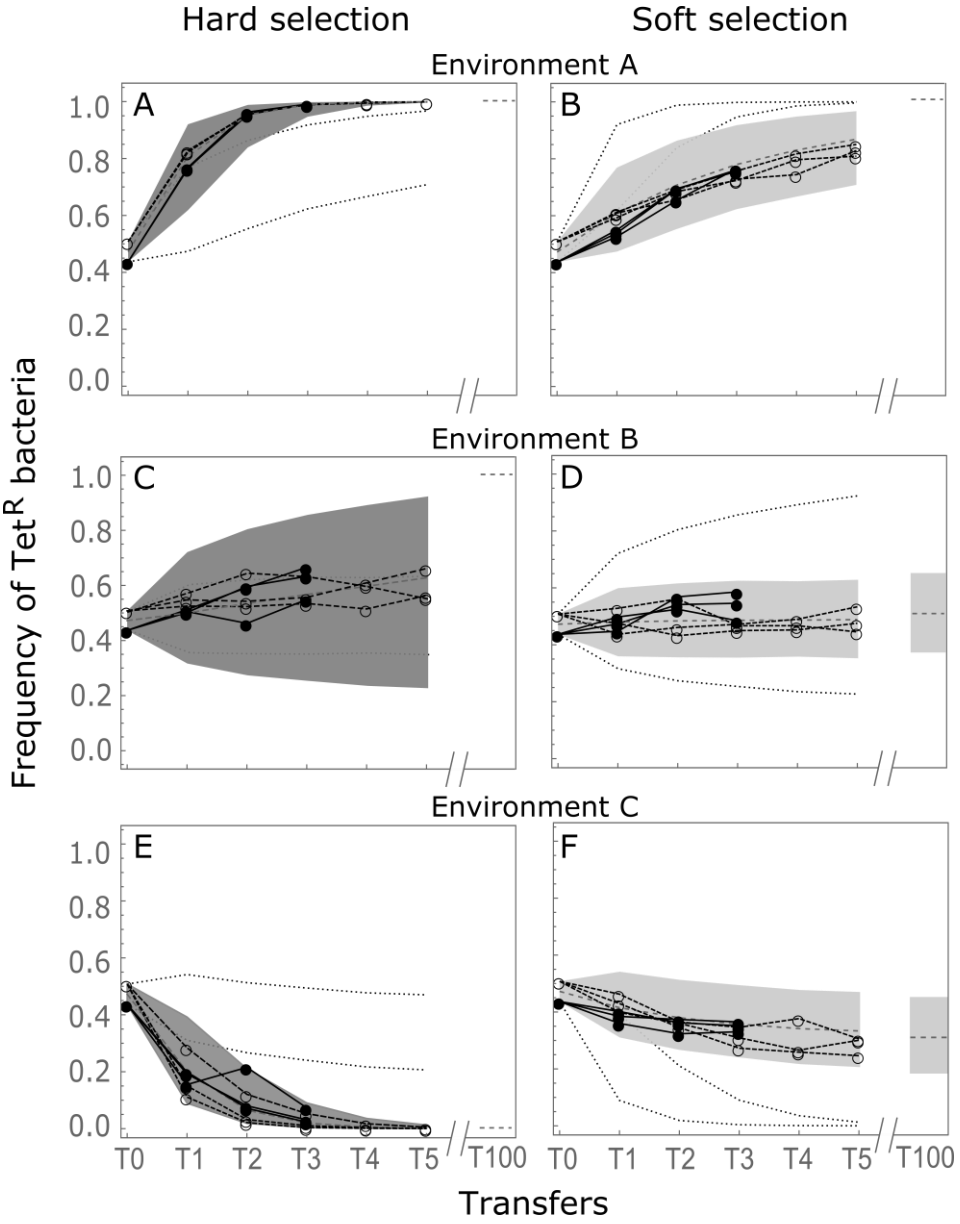

Figure 3: Evolution of genotype frequencies under hard and soft selection. All panels show the dynamics of the frequency of Tet^R bacteria over successive transfers in Experiment 1. Filled circles and lines show the frequencies observed in the three transfers of the short trial. Open circles and dashed lines correspond to the five transfers of the long trial. Note that initial frequencies slightly differ between the two trials. Grey areas show the 95% envelopes of theoretically predicted frequencies under the corresponding selection regime. Dashed grey lines show the medians of theoretical frequencies. Dotted black lines delimit the 95% envelopes of the other selection regime (e.g. on the hard selection panels, dotted lines show the predictions under soft selection). At the right of the x-axis break, theoretically predicted equilibrium frequencies are shown. Panels: A. Environment A (Nal2-Tet50) under hard selection. B. Environment A under soft selection. C. Environment B (Nal50-Tet50) under hard selection. D. Environment B under soft selection. E. Environment C (Nal50-Tet2) under hard selection. F. Environment C under soft selection.

In Environments B and C where stable polymorphism was expected, genotype frequencies fit well with predictions obtained under soft selection (light grey areas in figures 3D and F). In the five-transfer trial, the frequency of Tet^R bacteria finally attained 0.484 ± 0.045 (expected value: 0.488 with 95% envelope [0.353-0.628]) in Environment B and 0.284 ± 0.033 (expected value: 0.333 with 95% envelope [0.206-0.470]) in Environment C.

Polymorphism protection observed in soft but not in hard selection

The trajectories of genotype frequency observed in Experiment 2 were again in agreement with theoretical expectations (Figure 4). In Environment A (Figure 4A), as observed previously, neither hard selection nor soft selection produced an advantage for the rare genotype. In Environment B the Tet^R genotype had a global fitness advantage over the Nal^R competitor (Figure 4C). Tet^R frequency nevertheless significantly decreased from a high initial starting value under soft selection. Similarly in Environment C (Figure 4D), where Tet^R bacteria had a global fitness disadvantage, Tet^R frequency significantly increased when initially rare under soft selection only. In these two environments the observed polymorphism was therefore protected under soft selection.

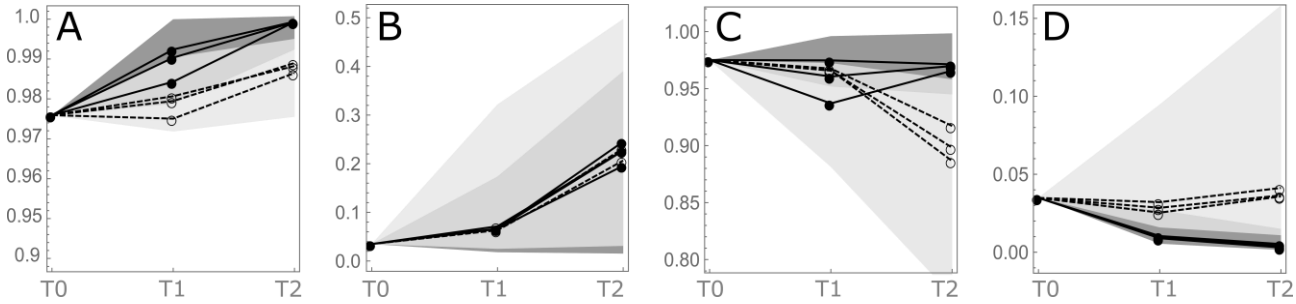

Figure 4: Polymorphism protection. All panels show the dynamics of the frequency of Tet^R bacteria over successive transfers in Experiment 2. Filled circles and lines show the frequencies observed under hard selection. Opened circles and dashed lines correspond to soft selection. Dark and light grey areas show the 95% envelopes of theoretically predicted frequencies under hard and soft selection, respectively. Panels: A. Environment A (Nal2-Tet50) with initially frequent Tet^R bacteria. B. Environment B (Nal50-Tet50) with initially rare Tet^R bacteria. C. Environment B with initially frequent Tet^R bacteria. D. Environment C (Nal50-Tet2) with initially rare Tet^R bacteria.

DISCUSSION

To our knowledge, the present study is the first conclusive experimental demonstration of a prediction that traces back to Levene (1953) and Dempster (1955): the way populations redistribute among habitats of a given environment is crucial for the long-term maintenance of local adaptation polymorphisms. Soft selection, in which habitat contribution to the next generation is constant, can protect polymorphism by producing negative frequency-dependent selection. In contrast, hard selection, in which habitat contribution to the next generation varies with the habitat genetic composition, never does so (*e.g.*, Christiansen 1974, 1975, Karlin and Campbell 1982, de Meeûs *et al.* 1993).

In the present study hard and soft selection were applied to populations composed of two bacterial genotypes in heterogeneous environments composed of two habitats. Bacterial genotypes and habitats were designed using antibiotic resistance so that each genotype was locally adapted to one habitat. The use of very low antibiotic concentrations was crucial. High antibiotic concentrations would have completely inhibited the growth of susceptible competitors, while very low concentrations simply provide a small fitness advantage to the resistant genotype. Although both genotypes could develop in both habitats, each genotype was specialist of one habitat. It was verified that habitats imposed a local adaptation trade-off that was not circumvented by bacterial evolution occurring during the experiment. This precaution was important as the absence of a clear local adaptation trade-off had led previous experimental tests of hard and soft selection to inconclusive results (Bell, 1997; reviewed in Vale, 2013). In the absence of a local adaptation trade-off, selection is expected to favor a single generalist genotype and environmental heterogeneity cannot lead to stable polymorphism maintenance. In nature, even in absence of a local adaptation trade-off, some polymorphism can be observed over long periods of time. Two underlying causes can be identified. First, transient polymorphism is less efficiently removed under soft selection than under hard selection (as observed in Bell, 1997, Jasmin and Kassen 2007). Second, negative frequency-dependence caused by factors other than environmental heterogeneity could be at work (*e.g.*, Hori 1993, Sinervo and Lively 1996,

Gigord *et al.* 2001, Olendorf *et al.* 2006). Discerning whether observed polymorphisms were due to the negative-frequency dependence produced by environmental heterogeneity or not was therefore of prime importance. This was done in two complementary manners. First, theoretical predictions accounting for experimental variability in habitat quality were produced and systematically compared to the observed genotype frequencies under hard and soft selection. Second, a complementary trial testing for an advantage of the rare genotype was conducted by applying soft and hard selection onto populations where the genotype with a disadvantage at the scale of the environment was rare.

In all treatments, experimental results showed a remarkable similarity to theoretical predictions. It was shown that hard selection does not protect polymorphism. The fixation of the genotype with the highest mean fitness at the scale of the environment was observed within 3 transfers in the two asymmetric environments. In the symmetric environment, polymorphism was still observed after 5 transfers under hard selection. But deviations to frequencies theoretically expected under soft selection (Figure 3) and the polymorphism protection experiment (Figure 4) confirmed that such polymorphism consisted of transient polymorphism not being easily removed because of very similar initial frequencies and local fitnesses. In contrast, under soft selection, polymorphism was never lost over the course of the experiment, even in asymmetric environments where the genotype adapted to the most productive environment had a mean fitness advantage. This was verified using theoretical predictions and the complementary experiment that such polymorphism was only transient in one of the two asymmetric environments, and that it was effectively protected by the existence of a systematic advantage of the rare (*i.e.*, negative frequency-dependence) in the two other environments. In these situations, even though one genotype has a higher mean fitness at the scale of the environment, the local regulation step that occurs at each transfer opposes the effect of within-habitat selection and hampers invasion of the whole environment by the genotype adapted to the most productive habitat (Levene 1953). Lastly the experiment confirmed that with all else being equal, even when soft selection is expected to lead to the fixation of a single genotype (*i.e.*, when its mean fitness at the scale of the environment is very high – environment A in figure 3), soft selection leads to a slower rate of evolution than hard selection (as shown by *e.g.*, Whitlock 2002).

The present experiment departed from real-world dynamics by using engineered bacteria in controlled environments. Transfers were controlled to reproduce the density-regulation steps characteristic of hard and soft selection. In contrast, between transfers, population growth processes (including birth and death) within environments were left uncontrolled over 8 to 10 generations per transfer. Nothing impeded the occurrence of complex population dynamics or density-dependence within habitats. Bacterial populations could for instance reach their carrying capacity before transfers, so that density regulation could be at work within habitats. In principle, this phenomenon is compatible with hard and soft selection. Local growth within habitats is represented in hard and soft selection models as local viabilities, *i.e.*, multiplication rates computed between transfers. The present experiment confirmed that the effects of hard and soft selection at the whole-environment scale were robust to local dynamics.

From a theoretical perspective it is understood that the conditions for polymorphism maintenance under soft selection are rather stringent (Prout 1968, Christiansen 1974, Maynard Smith and Hoekstra 1980). Various processes, such as drift and mutation, may reduce the range of parameters (trade-off shapes and habitat frequencies) where polymorphism is protected. This suggests that soft selection may not be that frequent in nature and that most observed polymorphism is either transient or maintained by other frequency-dependence mechanisms (de Meeûs *et al.* 2000). To some extent, the present study contradicts this view and suggests that the importance of soft selection in shaping standing genetic variation should not be overlooked (Agrawal 2010, Reznick 2016). In recent experiments, the 'softness' of selection (*i.e.*, the contribution of soft selection) was measured in experimental populations of both *Drosophila melanogaster* with different genes (Laffafian *et al.* 2010, Ho and Agrawal, 2014) and on seedling emergence in *Brassica rapa* (Weis *et al.* 2015). In addition to highlighting an unexpected sensitivity of softness to genes, individuals and population densities, it was found in both cases that the softness of selection was generally high, cementing the idea that soft selection shapes natural variation at local adaptation loci (Agrawal 2010, Reznick 2016).

The present experimental work provides new perspectives for further testing theoretical predictions about the effect of spatial heterogeneity on polymorphism maintenance. For instance, the present experiment conservatively considered full migration between the two habitats. But an important body of knowledge has explored the effect of migration between habitats on the conditions for polymorphism maintenance. Migration intensity (*e.g.*, Maynard

Smith 1966), timing (*e.g.*, Ravigné *et al.* 2004, Débarre and Gandon 2011, Massol 2013), and bias (density dependent migration or habitat selection, *e.g.*, de Meeûs *et al.* 1993) affect the range of conditions favorable to polymorphism maintenance and could be tested through a similar experimental design. Estimating the relative importance of spatial and temporal variability of the environment in shaping polymorphism could also help our understanding of ecological specialization (Massol 2013).

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Authorship statement

RF had the original idea of the study; RF, RG and VR designed the experiments; RF and RG carried out the experiments; RG analyzed experimental results; VR provided theoretical predictions; RF, RG and VR wrote the paper.

Data accessibility statement

The data supporting our results will be archived in an appropriate public repository (Dryad/Agritrop) and the data DOI will be included at the end of the article, upon acceptance on the present manuscript.

Competing Financial Interests statement

The authors declare no competing financial interests.

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SUPPLEMENTARY MATERIALS

Table S1. Statistical analysis of the relative genotype frequency variations. Relative frequencies were transformed with a logit function (), in order to linearize the curves and therefore perform linear regressions. R^2_{model}= 0.975.

Traitement	Selection	Estimate	Std. Error	t value	Pr(>|t|)
A	Intercept	0.01026	0.06951	0.148	0.883
	Hard	1.44043	0.03695	38.98	$<2.10^{-16}$
	Soft	0.32782	0.03695	8.871	2.73E-14
B	Hard	0.08501	0.03695	2.3	0.0235
	Soft	-0.0289	0.03695	-0.782	0.4359
C	Hard	-1.39113	0.03695	-37.646	$<2.10^{-16}$
	Soft	-0.21988	0.03695	-5.95	3.89E-08

Figure S1. Evolution of selection coefficients (*s*) during the experiment. Graphs on the left side show the evolution of *s* in the Nal-habitat, while graphs on the right side show the evolution of *s* in the Tet-habitat. It is to be noted that when relative frequencies reach extreme values (close to 0 or 1), the estimation of *s* are less precise, due to the detection of only few individuals of the losing genotype. This explains some s variations at transfer 5 in treatments A and C, and more specifically why we observed a very low *s* measure in the Nal habitat at transfer 5, in one population in treatment C.