Temperature effects on evolutionary rates of *Escherichia coli* with a growth advantage in stationary phase (GASP) phenotype

Master Thesis by Peter Schmid

Institute of Evolutionary Biology and Environmental Studies
University of Zurich
Winterthurerstrasse 190, CH-8057 Zurich

Supervised by Prof. Dr. Owen Petchey,
Prof. Dr. Andreas Wagner
and Sinisa Bratulic

Abstract

Adaptation of organisms to a rapidly changing world due to climate change requires more detailed knowledge on the speed of evolution. In this context, I investigated how temperature influenced the speed of evolutionary adaptation of *Escherichia coli* and how this could help to explain global patterns such as the latitudinal gradients of biodiversity. *E. coli* kept in stationary phase of population dynamics evolve a growth advantage in stationary phase (GASP) phenotype. I studied how population dynamics and evolution of the GASP phenotype were affected by temperature ranging from 32°C to 42°C. Densities of *E. coli* cells were counted by plating them on agar plates containing Luria-Bertani medium. The densities declined faster at higher temperatures after the cells acquired their maximal population density at carrying capacity. Populations grown at lower temperatures showed GASP over those grown at higher temperatures. There were some unexpected effects of aspects of the experimental design. Population dynamics and the GASP phenotype in my experimental conditions were in agreement with previously reported data. Nevertheless, it remains unclear why populations grown at lower temperatures showed GASP over those grown at higher temperatures. I also discuss how my findings relate to other research done on *E. coli* in stationary phase.

Table of contents

Introduction

Evolution and latitudinal gradients

The theory of evolution has been widely accepted as the most plausible explanation for the diversity of species one can observe today. This diversity of species is the result of the complex evolutionary history of our planet. Evolution occurs when natural selection acts on existing genetic variance by selecting phenotypes that have a higher fitness and are adapted adequately to the environment. If there are more speciation events than extinction events, biodiversity will increase.

Any kind of gradient in biodiversity might be a result of these two processes. For example, marine zooplankton diversity decreases in higher latitudes and lower sea surface temperatures [1]. This trend persists in a dataset of four different eras: 3 million years ago, 120'000 years ago, 18'000 years ago and today [1] (Fig. 1).

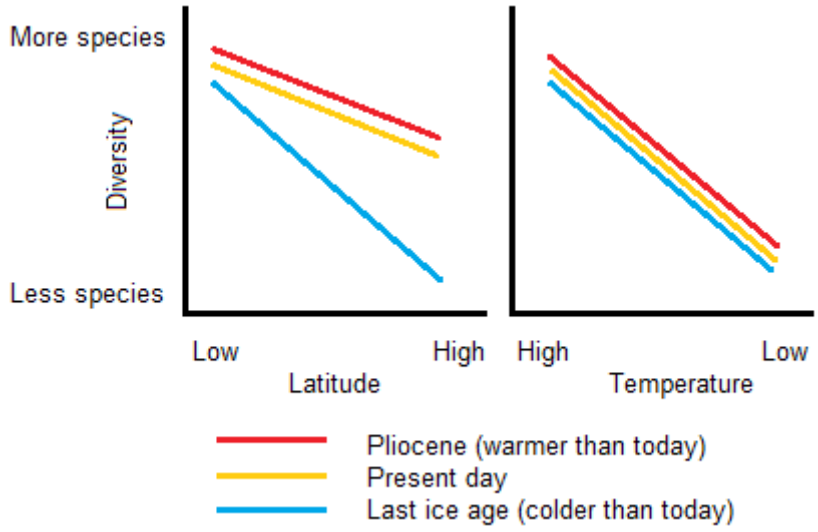

Figure 1: **Simplified graph showing marine zooplankton species diversity gradients.** The effect of latitude on the zooplankton diversity is much stronger during the last ice age. Temperature has a similar effect for each of the three time slices. Figure adapted from [1].

Biodiversity is generally lowest in Arctic biomes close to the poles and increases towards the equator [2]. Two main reasons have been proposed to explain this gradient. Firstly, higher temperatures are generally correlated with a higher energy input on the surface area. Therefore, primary production is mostly higher in tropic regions. This supports more organisms and a higher proportion of specialists [3]. The second reason is the influence of temperature on metabolism. Metabolic rates increase exponentially with temperature. As the metabolic rates correlate with mutation rates and generation time, a higher metabolic rate causes more speciation events [2, 4].

Thus, higher temperatures result in higher rates of evolution and should lead to increased biodiversity.

The strength of the latitudinal gradient varies among ecosystems and does not solely depend on metabolism. For example, the diversity of tree species decreases faster from south to north in eastern Asian forests compared to forests on North America [5].

Metabolic rates, temperature, and evolutionary rates

What examples support the theory that metabolic rates correlate positively with evolutionary rates? Mitochondrial and nuclear substitution rates in turtles [6] have been shown to correlate negatively with latitude, and thus positively with temperature, rather than with life-history factors. By studying speciation events and DNA evolution of global datasets from planktonic foraminifera spanning thirty million years, Allen et. al. [7] found that speciation rates were higher in tropic, thus warm, regions, than in regions further south/north. Species of parmelioid lichen clades occurring in oceanic and tropical regions, where it is warm and moist, showed higher rates of evolution than those living in semi-arid and temperate regions [8]. A low variation of mitochondrial DNA might indicate a slow molecular evolution in Arctic fishes living at temperatures below zero because of decreased metabolic rates [9]. These examples support that metabolic rates influence evolutionary rates because they correlate positively with temperature.

***Escherichia coli* and the growth advantage in stationary phase (GASP)**

In the model species *Escherichia coli* (Fig. 2), the temperature dependence of metabolic rates is relatively constant for temperatures ranging from 0°C to 40°C [10]. *E. coli* grow optimally at 37°C and they are thermal generalist within their growth spectrum [11]. *E. coli* can rapidly evolve to be adapted to higher temperatures such as 42°C [12].

When *E. coli* cells have to cope with scarce resources, they evolve a phenotype called growth advantage in stationary phase (GASP). If a culture of *E. coli* is left to starve for a few days, the bacteria will respond to the starvation by slowing down the metabolism and by increasing their resistance to environmental factors [13]. Evidence of GASP can already be observed after ten days of evolution in a stationary phase environment: aged cultures overtake their ancestors when they are mixed together [14].

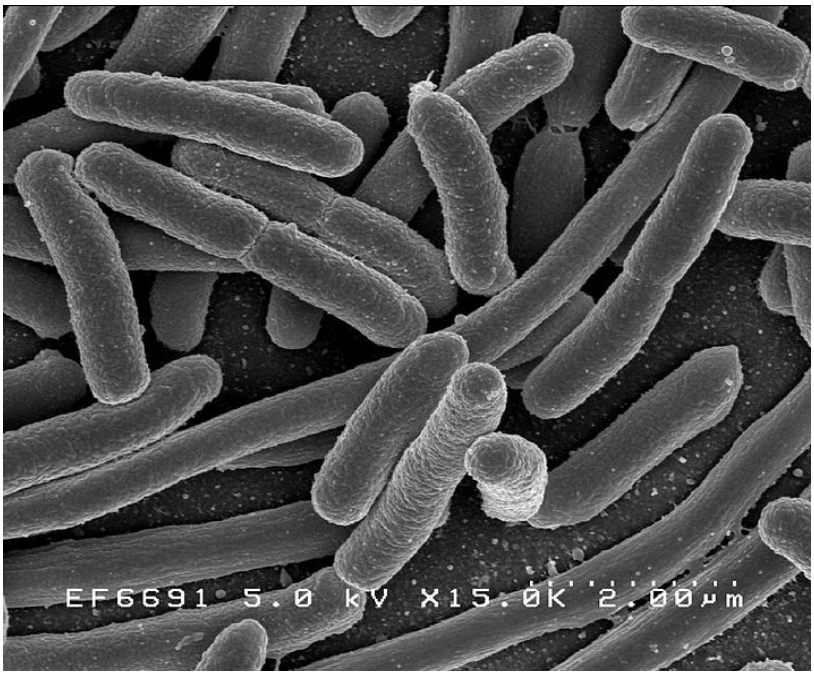

Figure 2: **Electron micrograph picture of E. coli cells.** Source: http://en.wikipedia.org/wiki/File:EscherichiaColi_NIAID.jpg

On fresh Luria-Bertani medium (LB medium), *E. coli* cells will go through five phases in their population dynamics unless there are later inputs of nutrients to the system (Fig. 3). The population dynamics begin with a short lag phase where the cells do not grow or divide. Next is the exponential phase during which cells are not nutrient limited. As nutrients are depleted, population growth slows until the carrying capacity is reached. For about two days, the population density remains at carrying capacity in stationary phase. Thereafter, 1% of the *E. coli* culture survives the death phase. The surviving cells are the foundation of the genetic variance that can now enter the long-term stationary phase and evolve the GASP phenotype [15]. *E. coli* cells use different DNA polymerases for each of these five phases and they time the usage of these polymerases independently, without exogenous agents [16]. Changing the polymerases gives advantages in genetic variation and optimal fitness [16].

Bacteria with the GASP phenotype have been shown to get out-competed by wild types in fresh medium [17]. There is a trade-off between being adapted to an environment with scarce resources, and fast metabolism and growth [17]. Population densities in the long-term stationary phase are approximately constant, but decrease slowly. Nevertheless, these population dynamics are not static as new GASP mutants evolve and out-compete older ones. This results in a constant change of the genetic composition of the population [15] (Fig. 4).

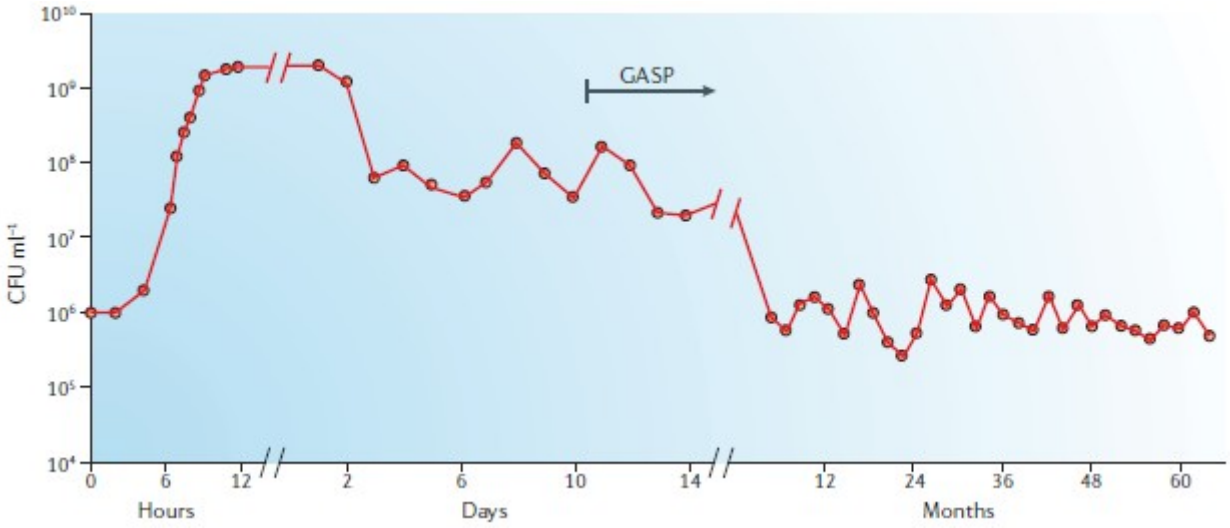

Figure 3: **The five phases of population dynamics in *E. coli*.** CFU: Colony forming unit. After a short lag phase, the bacteria go through exponential growth and stay at the carrying capacity until about 1% survived the death phase. After that, the long-term stationary phase can continue for years and is characterized by a slow decrease in population density. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology [15], copyright 2006.

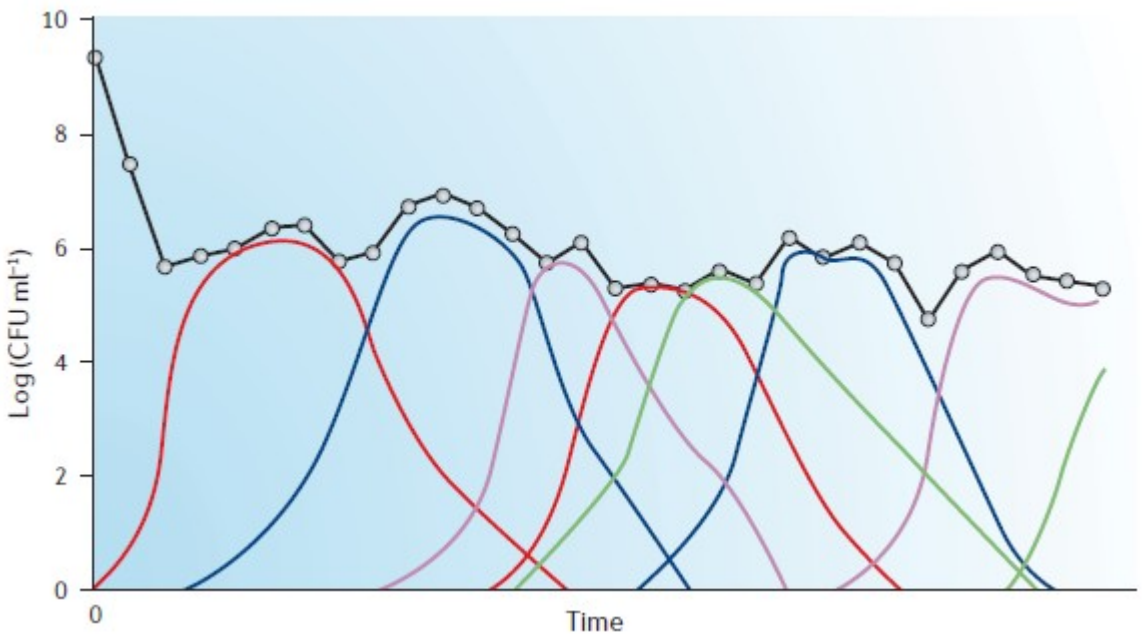

Figure 4: **Dynamics of the long-term stationary phase.** Although the overall population density is approximately constant, sub-populations of new mutants arise and become the majority while older GASP mutants decrease in density. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology [15], copyright 2006.

New GASP mutants have a competitive advantage when they are at low frequency in the population [18]. These mutants affect the wild type (compared to the new mutant) adversely, because they do not maintain the cooperative non-dividing state of the wild type, where cells do not divide or very rarely, and increase in density. The mutant cells act differently and use an egoistic strategy that profits from the cooperative strategy of not dividing during stationary phase the wild

type uses and effectively replaces the wild type. This explains the many population shifts during the long-term stationary phase [18].

Mutation rates differ from stationary phase to growth phase. Adaptive mutations occur solely in non-dividing or slowly growing cells in stationary phase and when they are selected for [19]. For example, an *E. coli* lac(-) strain evolved several lac(+) colonies when grown on medium containing lactose [20].

Although a generation in stationary phase is estimated to be within 12 to 72 hours, cells living in stationary phase can accumulate many mutations, because their response to starvation induces mutagenesis and this response yields more mutations compared to the phases with frequent cell divisions [21]. Mutation rates in stationary phase are likely influenced by several factors, such as temperature-mediated biochemical reactions, the strain genotype and the population dynamics of different mutants that live in the stationary phase [21].

The starvation, the frequent turn-over of new mutant populations and increased mutation rates lead to a high genetic variance that can be maintained for years [22]. The amount of diversification positively correlates with the population size and initial resource concentrations of *E. coli* cultures [23].

The GASP mechanism

What are the mechanisms that allow the starved *E. coli* cells to maintain a population in long-term stationary phase? Bacteria utilize a horizontal gene transfer and exchange DNA. An environment containing few resources forces cells to use the energy from the DNA of lysed cells. Survivor cells metabolise that DNA [24]. There are more prerequisites, mostly genetic variation, to survive and out-compete other variants in stationary phase: mutations in the gene RNA polymerase sigma S (RpoS) were the first mutations found to be directly linked to the GASP phenotype [14]. The sigma factor of RpoS controls the general stress resistance of *E. coli*. Cells that mutate their RpoS regulon partially lose stress resistance. Simultaneously, the cells induce a response to starvation by increasing their effort to scavenge nutrients. These changes in the sigma factor of RpoS enables them to cope with the stationary phase conditions [25]. A micro-array analysis of genes regulated by RpoS has shown that more than a hundred genes are up- or down-regulated by RpoS and that some of the down-regulated genes could play an important role for the GASP phenotype [26].

RpoS is not the only global regulator responsible for the GASP phenotype. Mutants containing a GASP promoting mutation in the lrp regulon out-competed aged culture cells that already evolved changes in RpoS to deal with the stationary phase conditions [27]. Both RpoS and lrp lead to GASP when mutations trigger a loss-of-function for at least parts of the regulons [14,

27]. Another GASP regulator is the bgl operon that is usually silent in wild type *E. coli*, but mutations that activate this operon are advantageous in a stationary phase environment, because it allows them to metabolize aromatic (beta)-glucosides such as salicin and arbutin [28]. Many regulons are known to influence the expression of the bgl operon. For example, the bgl expression increases when the allele RpoS819, a known GASP allele, is present. Interactions between these regulons and operons seem to be the reason why bgl is much more active in stationary phase than during growth phase [29].

In my master thesis, I used the GASP system of *Escherichia coli* at a temperature range from 32°C to 42°C to evolve mutants that grew at different temperatures. The aim was to compare different evolutionary times and temperatures as conditions under which the bacterial populations evolved. These comparisons can be used to quantify the GASP ability of different populations and reveal how temperature influences the rate of evolution. To my knowledge, this is the first work that tries to find out, how temperature influences evolutionary rates in an experiment.

To answer questions regarding the effect of temperature on the rate of evolution based on the GASP system, I have formulated the following hypotheses:

1. The *E. coli* cultures should follow the phases of population dynamics and decrease in density during the experiment. I hypothesize that the decrease of the population density will be faster at higher temperatures. Metabolic rates correlates positively with temperature and the upper end is close to the growth range of *E. coli*. Therefore, when the nutrients get scarce, population densities should decline faster at higher temperatures.

2. The evolved *E. coli* populations should out-compete ancestors in a competition trial. The longer populations had time to evolve the GASP phenotype, the more clearly they should out-compete the ancestors. I expect to see evolution of GASP, as has been observed before [14].

3. In competition trials between strains with the same time of evolutionary history, those evolved at 37°C are expected to out-compete those evolved at 32°C and 42°C. Populations grown at 37°C are expected to evolve the fastest, because it is their growth optimum and generation times are the shortest.

Materials and Methods

Equipment

I used incubators of the series ILW 115 TOP from Pol-Eko Aparatura (Poland, ul. Kokoszycka 172C, 44 - 300 Wodzisław Śląski) for the growth of liquid cultures during the evolution experiment and the competition trials. Plates were inoculated in an incubator SGMIR-154 from Panasonic (Japan, 1006, Oaza Kadoma, Kadoma-shi, Osaka 571-8501). Shakers were bought from Phoenix instrument, series RS-OS 10 (Germany, Heinkelstr. 10 Garbsen 30827).

Bacterial strain description and growth conditions

I used the *E. coli* K-12 F- (non-mating) strains ZK1142 (nalidixic acid resistant, mutation on gyrA) and ZK1143 (streptomycin resistant, mutation on rpsL), which have the same genome except for the respective antibiotic resistance gene [22]. The strains were received from Steven E. Finkel (University of Southern California, Los Angeles) and are described in another paper [14]. A previous study verified the strains for spontaneous mutations that would allow one strain to survive on the other antibiotic [22]. Cultures grew in 10ml of LB medium without antibiotics in 15ml falcon tubes. They were kept on shakers rotating at 400rpm and with a slight angle downwards to allow more gas flow between the culture medium and the air in the incubator. Incubator temperatures were constant and I used eleven different temperatures ranging from 32°C to 42°C by 1°C. Every week, ddH20 was supplied until there was 10ml of liquid in each of the falcon tubes. The evolution experiment ran in three biological replicates and lasted six weeks.

Handling and measuring of the *E. coli* cultures

I sampled cultures weekly by freezing 100µl of each culture into a kryotube containing 150µl LB medium and 150µl glycerol. To count colonies, samples from the population were diluted and plated on LB agar plates. For the evolution experiment, plates did not contain antibiotics. Neither antibiotic affected the growth of *E. coli* if they had the respective antibiotic resistance gene.

Competition trials

Competition trials followed protocols used in previous GASP experiments [22]. To start a competition trial, I first grew overnight cultures in 15ml falcon tubes containing 10ml LB medium. After growing overnight, 10µl of one competitor were added to a culture containing the competitor of the other strain. Then I transferred 10µl from this tube back to the first, thus two cultures were

purposely cross-contaminated. The falcon tubes were vortexed each time before taking out a subset of the population. The strain with the higher initial density is called the majority and the other is called the minority. Before mixing the competitors together, plating part of the cultures onto LB agar plates containing antibiotics verified each tube for unintentional cross-contamination. After twelve days, I plated each competition trial to count the population densities. Initial densities are derived from the initial densities measured during the evolution experiment for one day old populations. The initial densities reflect the carrying capacity. To count densities from the competition trials, I plated several dilutions of the trials onto plates containing either 25µg/ml nalidixic acid or 50µg/ml streptomycin. All plates were incubated for 12 hours at 37°C, and then stored at 6°C until assessed (Fig. 5).

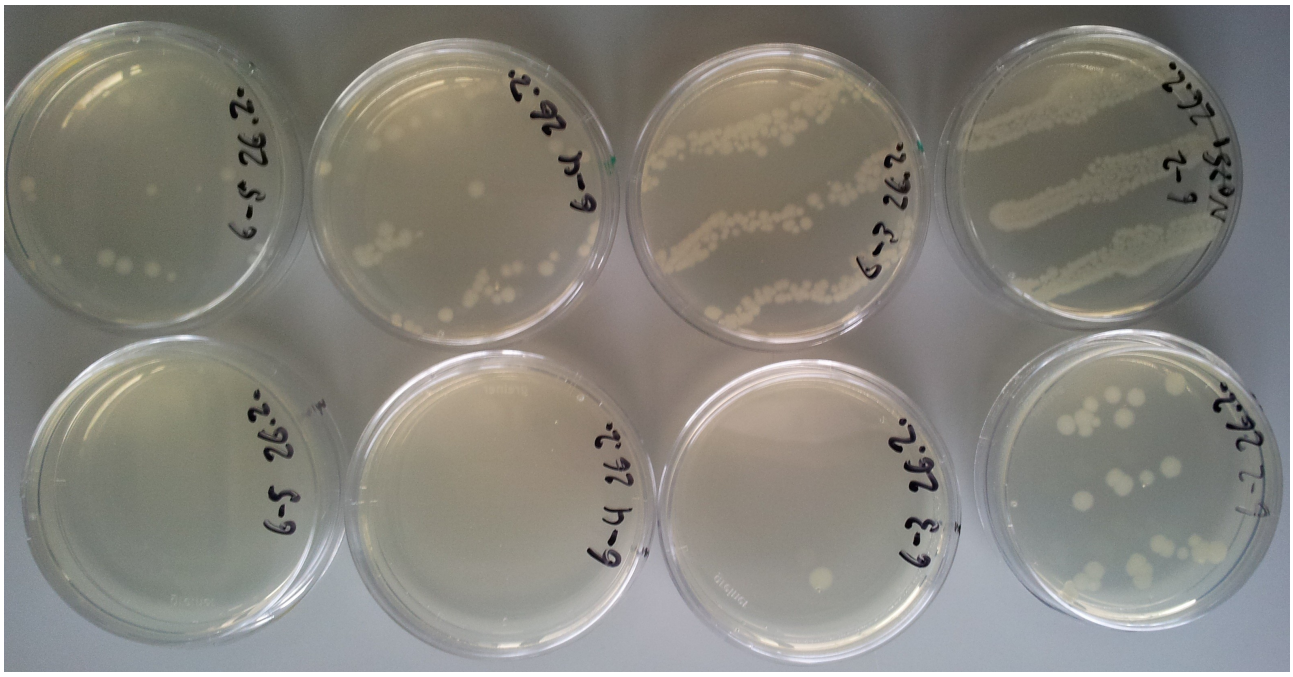

Figure 5: **Plates from a preliminary competition trial experiment.** The dilution increased by a factor of ten for each step from right to the left. The top row contained streptomycin and the bottom row nalidixic acid. The nalidixic acid resistant strain started as the majority, but got overtaken in population density by the other strain after twelve days of competition.

Competition trials were conducted between the two ancestral strains and six selected populations representing both strains and the three selected temperature levels: 32°C, 37°C and 42°C at different weeks of their evolutionary history. In one competition trial set-up, all six populations evolved until week 2, 3, 4, 5 and 6 competed against ancestors carrying the other antibiotic resistance gene. This first experimental set-up verifies if there is any evidence of GASP and whether the time the populations had to evolve influenced the amount of GASP they showed.

The other set of competition trials consisted of comparisons within the weeks of time the

population had to evolve and between the three temperatures. The strains from 37°C were tested against those from 32°C and 42°C. 32°C was not tested against 42°C (Fig. 6). The second experimental set-up investigated the effects of temperature on GASP ability and thus also on evolutionary rates.

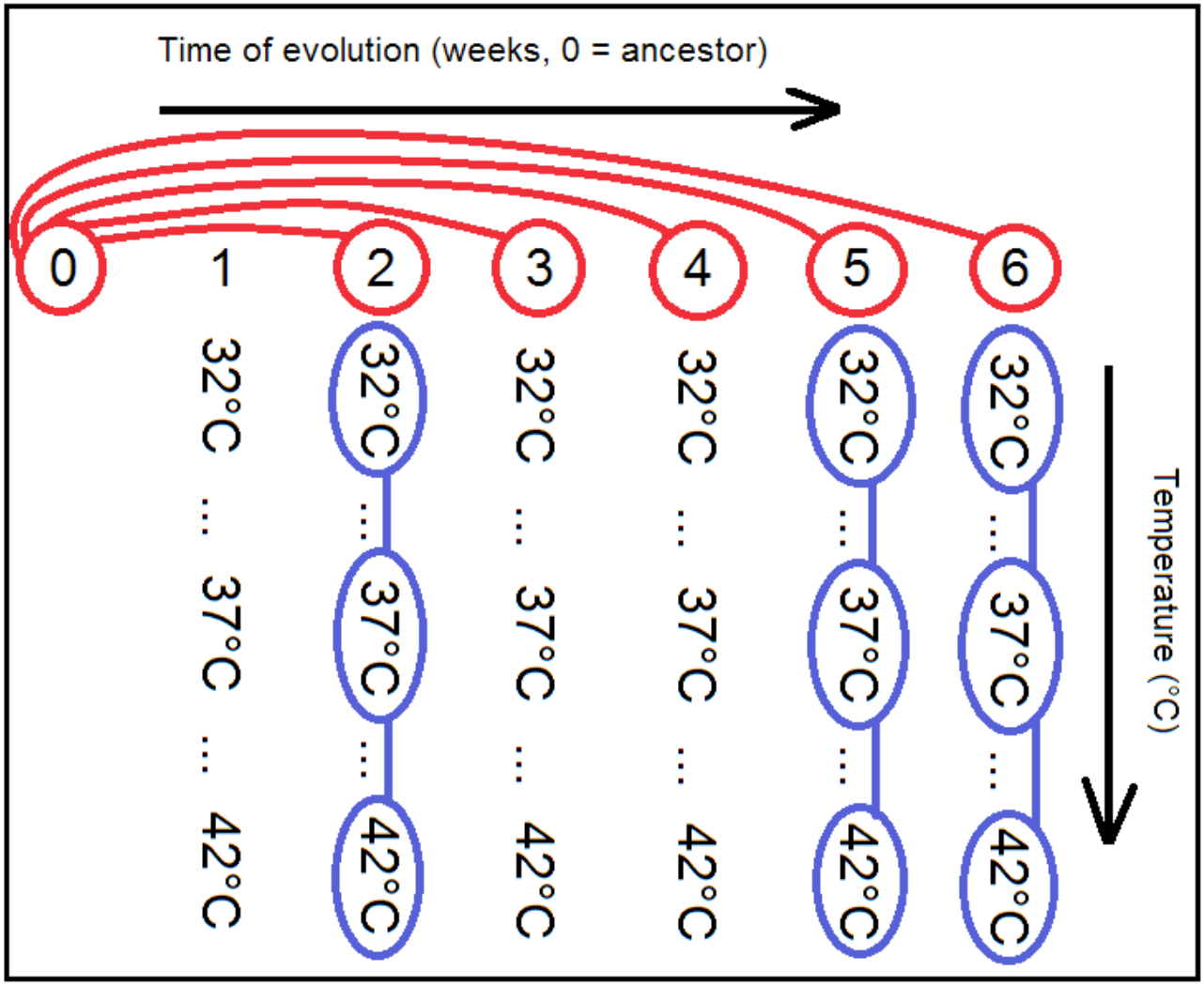

Figure 6: **Overview of selected competition trials.** Comparisons against the ancestor strains are shown in red and were done with the same six populations that were tested against each other shown in blue.

Experimental design and evaluation of competition trials

I quantified the outcome of competition trials to describe the difference in the density ratios of the competitors during the competition trials:

$$outcome = \log_{10}\left(\frac{A_{t0}}{B_{t0}}\right) - \log_{10}\left(\frac{A_{t12}}{B_{t12}}\right)$$

Where *A* is the strain that starts the trial in majority and *B* the one starting in minority. *t0* is the start point after the overnight cultures before the strains get mixed and *t12* is the end point after twelve days. Hence an outcome bigger than 0 means that the minority has increased relatively to the majority and an outcome less than 0 means the opposite.

A continuous linear model describes competition trials between weeks. For competition trials between temperatures, a binomial generalized linear model was used to account for not normally distributed data. Another reason to transform the data was that some outcomes were much further away from zero than most other outcomes (or all in the case of competition trials between weeks). These high differences in population densities occurred when one population went extinct while the other grew very well. As the exact extinction occurrence is not known, it might be possible that the survivor grew alone without competition. Therefore, I transformed the data by converting negative values into zeroes and positive values into ones.

Possible set-up combinations for competition trials depended on whether the competition trial was performed between temperatures or between weeks (one of the factor was always kept constant) (Fig. 7).

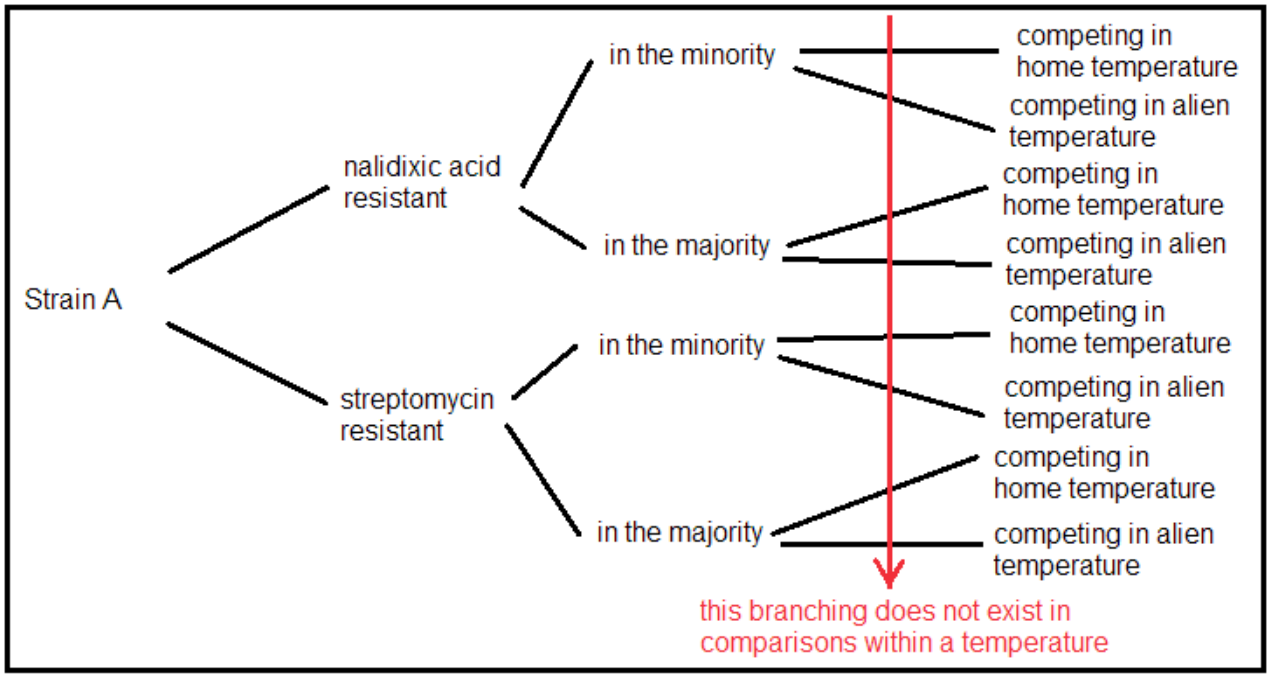

Figure 7: **Possible factorial combinations for one entire set of competition trials.** One true replicate of competition trials between temperatures consists of eight single competition trials. Competition trials conducted between competitors of the same temperature only consist of four competition trials for one true replicate.

Strain *A* can have either antibiotic resistance, start either as the majority or the minority, and in the case of comparisons between temperatures, that strain can either compete at the temperature

at which it evolved or at the temperature at which the competitor evolved. This increases the amount of effort per replication and therefore limited the number of conducted competition trials.

Statistical analysis

The statistical analysis was performed in R using the lattice and arm packages [30]. I analysed the results using linear models, generalized linear models and ANOVA.

Population densities of the evolution experiment were measured after 16 hours and thereafter each week for all populations. First, a linear model described the overnight densities at carrying capacity and verified whether these densities are useful for the competition trials:

$$\log_{10}\left(\frac{CFU}{ml}\right) \sim poly(temperature, 2) * strain \quad (1)$$

Where *CFU/ml* describes colony forming units per millilitre, a measure of population density and *poly(x,n)* is a polynomial of grade *n* for the factor *x*.

The densities from each week and the overnight densities were plotted. I used a phenomenological linear model to find significant effects and to accept or reject the first hypothesis regarding the population dynamics:

$$\log_{10}\left(\frac{CFU}{ml}\right) \sim temperature * poly(week, 3) * strain \quad (2)$$

Competition trials between weeks (against the ancestors) were analysed with a linear model:

$$quant \sim week * temperature * maj.ancestor * maj.strain \quad (3)$$

Where *quant* is the outcome of the competition trial, and *maj.ancestor* and *maj.strain* are both factors with two levels and are tied to whether the ancestor, or one of the strains, started in the majority or the minority.

Because all evolved populations were compared to two ancestral strains, the data points are dependent. Therefore, I randomized the response variables 999 times and ranked the model within these 1000 outcomes of the model. P-values were calculated from these ranks and compared to the p-values from the model.

Competition trials between temperatures were transformed into binomial data (i.e. positive

values become 1 and negative values become 0). The following binomial generalized linear model was used for these competition trials:

$$\begin{aligned} bin \sim\ & week + comp.temp.rel + maj.temp.rel + maj.strain + temps \\ & + all\ two-way\ interactions \\ & + week*comp.temp.rel*maj.temp.rel + comp.temp.rel*maj.strain*temps \end{aligned} \quad (4)$$

Where *bin* is the outcome of the competition trial after the data had been transformed into binomial data, *comp.temp.rel* indicates whether the competition trial was done in the higher temperature or in the lower temperature, *maj.temp.rel* indicates if the majority competitor was the one from the high temperature or the one from the low temperature and *temps* is a factor that differentiates between the two comparisons of 32°C versus 37°C and 37°C versus 42°C.

The replication was not high enough for all treatment combinations to use the full model. Thus, a less complex model was used that could explain as much deviance as possible while keeping the occurrence of fitted probabilities of 0 and 1 as low as possible. Many of the three-way interactions explained almost zero deviance and were thus excluded from the model.

Results

Contamination and missing values

All observed contamination events were cross-contaminations with either of the strains. This was apparent when a population could grow on plates containing one or the other of the two antibiotics. Five such cases occurred during the evolution experiment. These five populations were excluded from the analysis except for the analysis of the overnight densities, because there was no evidence of cross-contamination that early in the experiment.

Evolution experiment

The population densities differed only slightly after 16 hours and were in the range of 10^8.8 CFU/ml to 10^9.3 CFU/ml (Fig. 8).

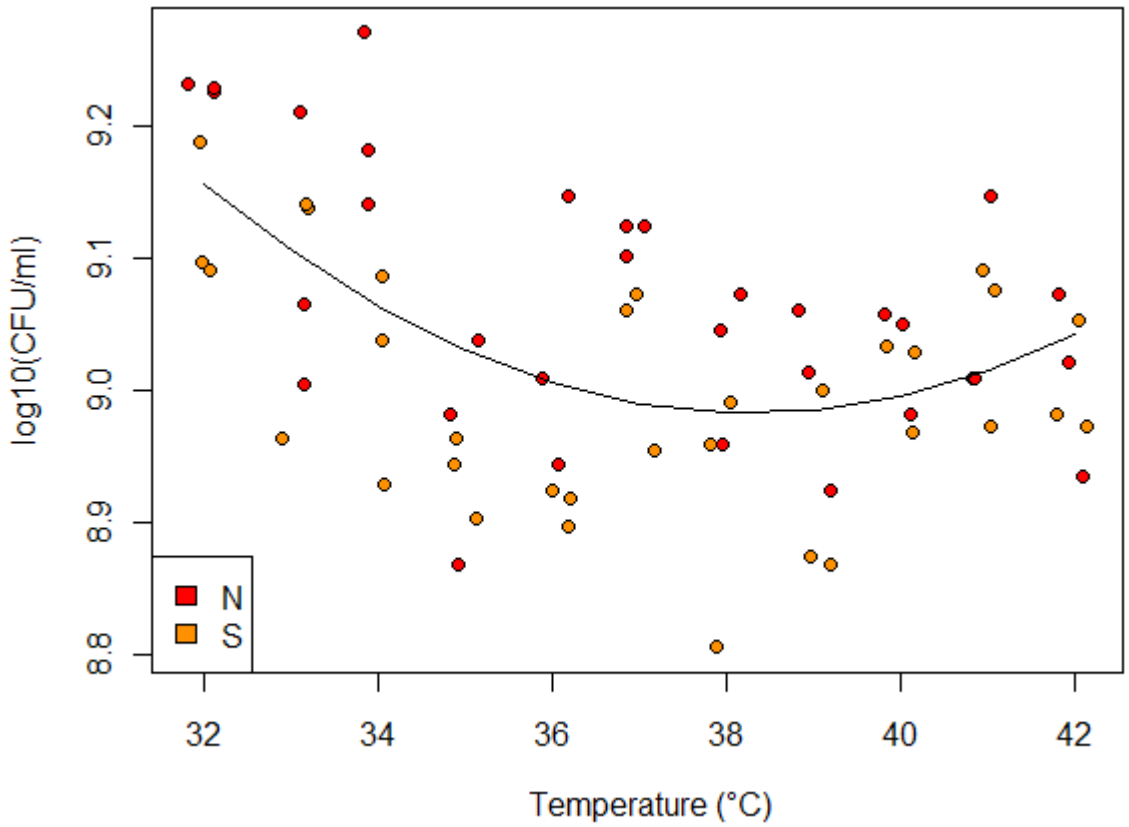

Figure 8: **Population densities of the evolution experiment after 16 hours.** N is the nalidixic acid resistant strain and S is the streptomycin resistant strain. The fitted line was predicted from a model containing only the second polynomial of the temperature. X-values of the the data are jittered to display all data points. Note the large difference of the Y-axis scale compared to the next plot.

The predicted curve through the overnight densities had its minimum density approximately at 38°C. The linear model (1) had a significant temperature effect on the linear regression (1st degree

polynomial: about -0.39 in log-density per 1°C, p-value < 0.001; 2nd degree polynomial: about 0.23 in log-density per 1°C, p-value < 0.05). The identity of the strain also played a significant role in the linear regression (p-value < 0.001). Both the temperature and the strain identity significantly explained variance in the ANOVA associated with the linear model (1) (p-value < 0.001 for both). The interaction term was not significant.

As the overall variance in the overnight densities was relatively low, these estimates were used for the initial densities of competition trials instead of measuring them directly. The overnight growth conditions were the same for the evolution experiment and the competition trials.

The population dynamics decreased faster at the first half of the experiment and slower at the second half. The decrease in density was faster and more extreme at higher temperatures (Fig. 9).

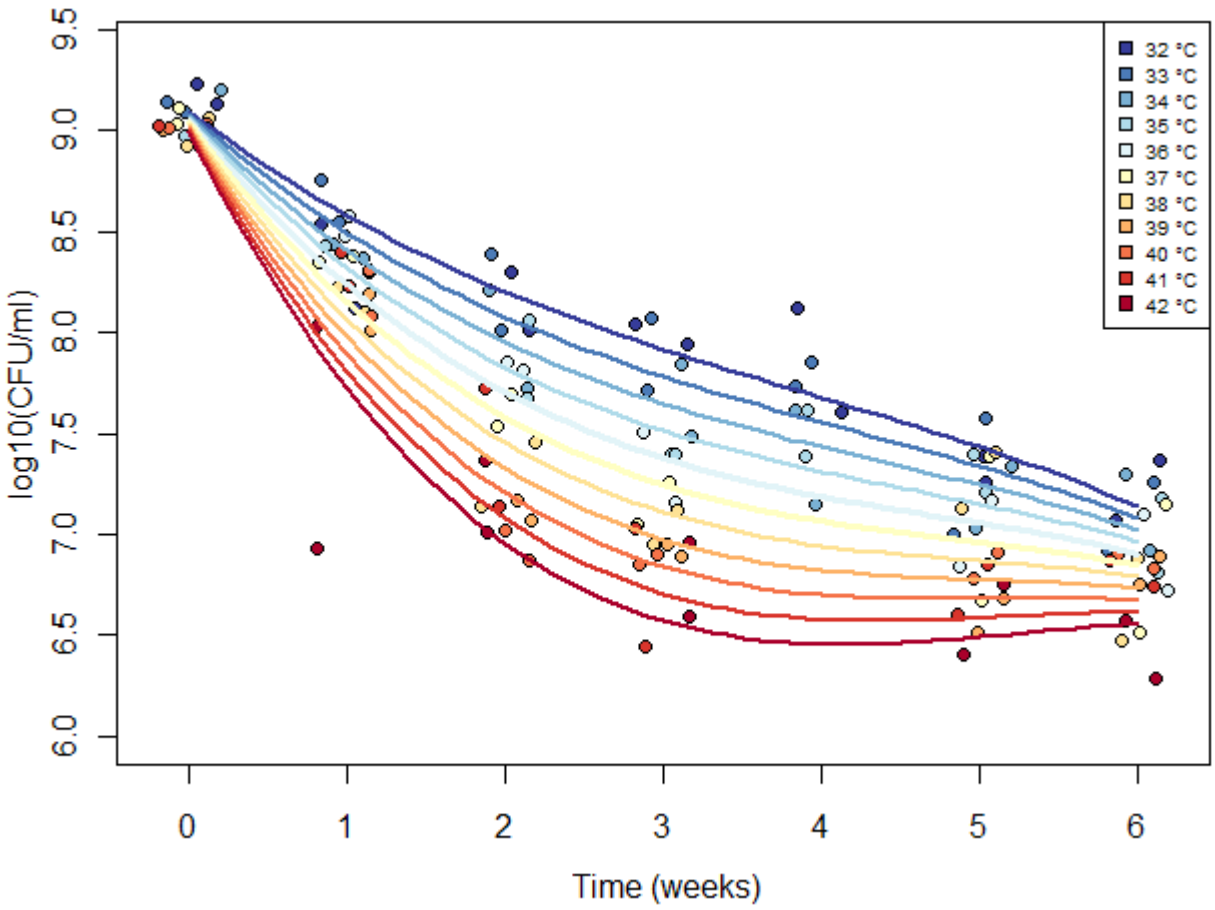

Figure 9: **Population dynamics of the evolution experiment.** Each dot represents the mean density of up to three replicates of that strain, temperature and week. The model used to predict the lines did not distinguish between the two strains to keep the plot well-arranged. X-values of the the data are jittered to display all data points.

The linear model (2) had a significant three-way interaction between the factors temperature,

strain identity and the second polynomial of the factor week (p-value < 0.05). The three-way interaction was not significant in the ANOVA of the linear model (2), but all three two-way interactions were (p-value < 0.01 for the temperature-by-strain interaction and p-value < 0.001 for the other two). Populations of the nalidixic acid resistant strain had slightly higher densities than those from the streptomycin resistant strain.

Competition trials between weeks (compared to the ancestor)

The results from the randomizations yielded similar values as the p-values estimated from the ANOVA of the linear model (3). Reported significances were true for both methods because of their similarity.

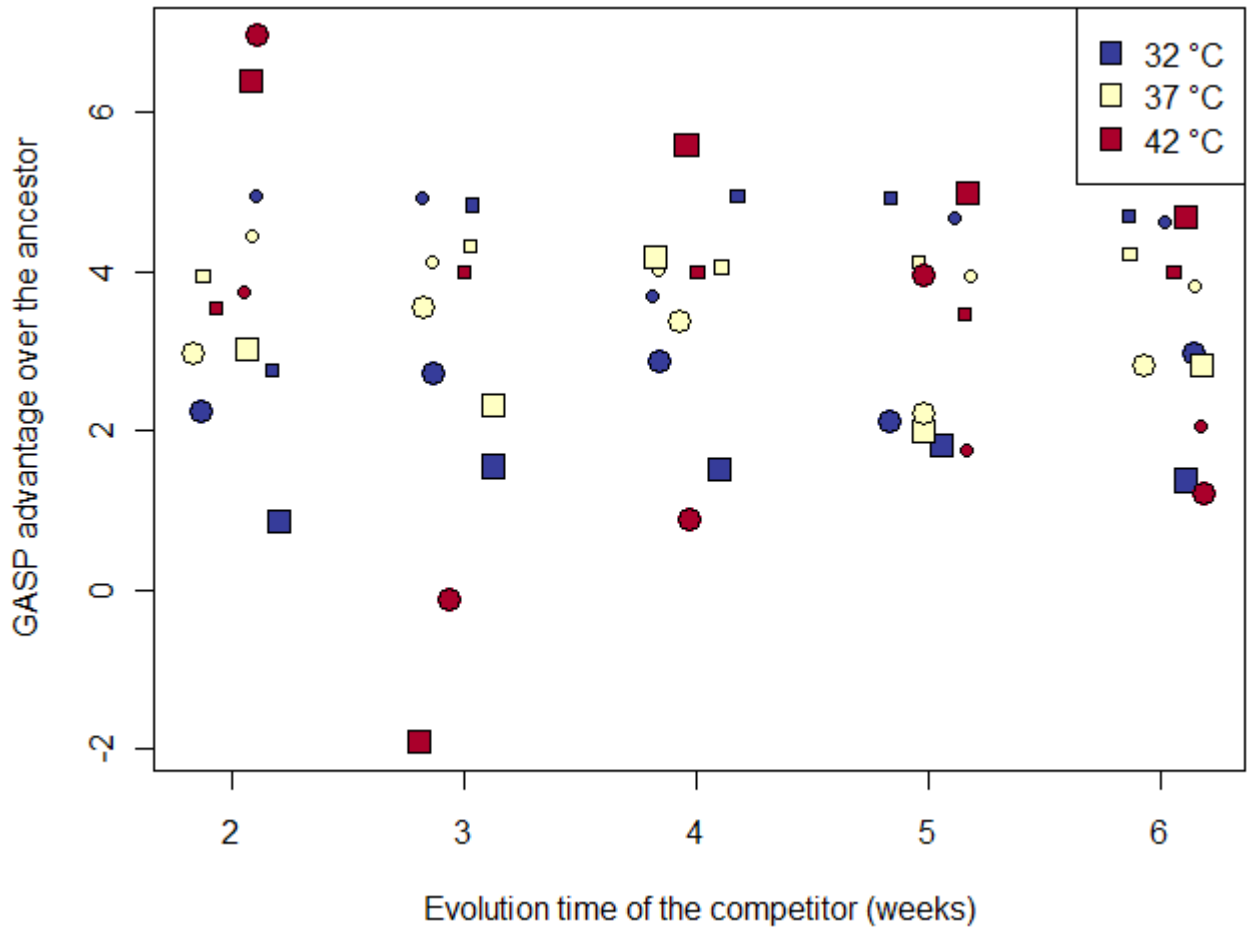

Figure 10: **Outcome of competition trials between weeks.** Big/small shapes show whether the ancestor started as the majority/minority, circles/squares indicate that the streptomycin resistant population started as the majority/minority. The colour shows the temperature at which the competition trial was performed and at which the mutant population has evolved. X-values of the the data are jittered to display all data points.

None of the coefficients of the linear model (3) was significant, but the corresponding

ANOVA showed a significant effect of the ancestral starting position (majority or minority, p-value < 0.001). There were two significant interactions between temperature and strain identity of the majority or the ancestral starting position (majority or minority, p-value < 0.05 for both). The evolution time of the evolved strain was not significant (Fig. 10).

The evolved populations out-competed the ancestors in 56 of 58 cases. Therefore, the graph clearly shows that almost all the evolved populations had a growth advantage in stationary phase over the ancestor. A simple binomial test using the proportion of wins and amount of trials yielded a 95% confidence interval of 0.881 to 0.996, which was the probability of the evolved population increasing in density relatively to the ancestor (Fig. 10).

Competition trials between temperatures

Different generalized linear models were fitted to the data, but none of them could remove the occurrence of fitted probabilities 0 and 1, because the separation of the data was extreme.

None of the coefficients of the generalized linear model (4) was significant, but the corresponding ANOVA showed that the factor *maj.temp.rel* had a significant difference (p-value < 0.001). The strain identity of the majority also played a significant role (p-value < 0.05) as well as its interaction with *maj.temp.rel* (p-value < 0.01). Other significant interactions were: week by strain identity (p-value < 0.01), *maj.temp.rel* by the pair of the competition trials (either 32°C vs 37°C or 37°C vs 42°C) (p-value < 0.01) and a three-way interaction consisting of the factors *comp.temp.rel*, *maj.strain* and *temps* (p-value < 0.01). Thus, all factors were included in at least one interaction (Fig. 11).

The generalized linear model (4) was then used to predict the 95% confidence interval of the strain that evolved at the lower temperature showing GASP. The lower temperature had a significantly higher chance to win in both cases. The 32°C population had a chance of 62.5% to out-compete the 37°C population (95% confidence interval: 56.7% to 68.3%) and the 37°C population had a chance of 68.8% to out-compete the 42°C population (95% confidence interval: 60.1% to 77.4%). In 57 out of 76 competition trials, the minority won. Without explaining more variance with a model, a simple binomial test shows that starting as the minority significantly improved the chances of winning (95% confidence interval: 63.7% to 84.2%) (Fig. 12).

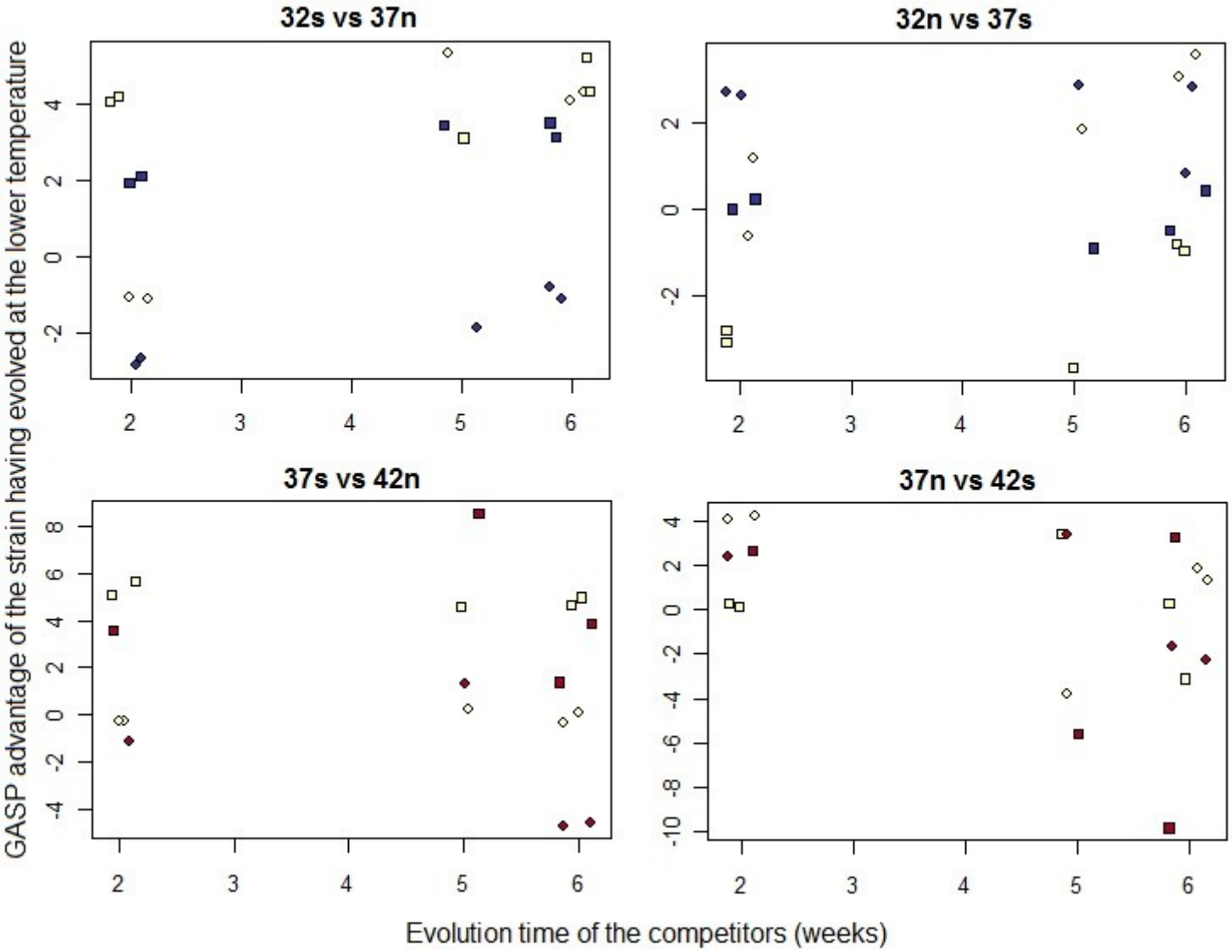

Figure 11: **Outcome of competition trials between temperatures.** Circles/squares indicate that the streptomycin resistant population started as the majority/minority. Blue corresponds to 32°C, yellow to 37°C, and red to 42°C. X-values of the the data are jittered to display all data points.

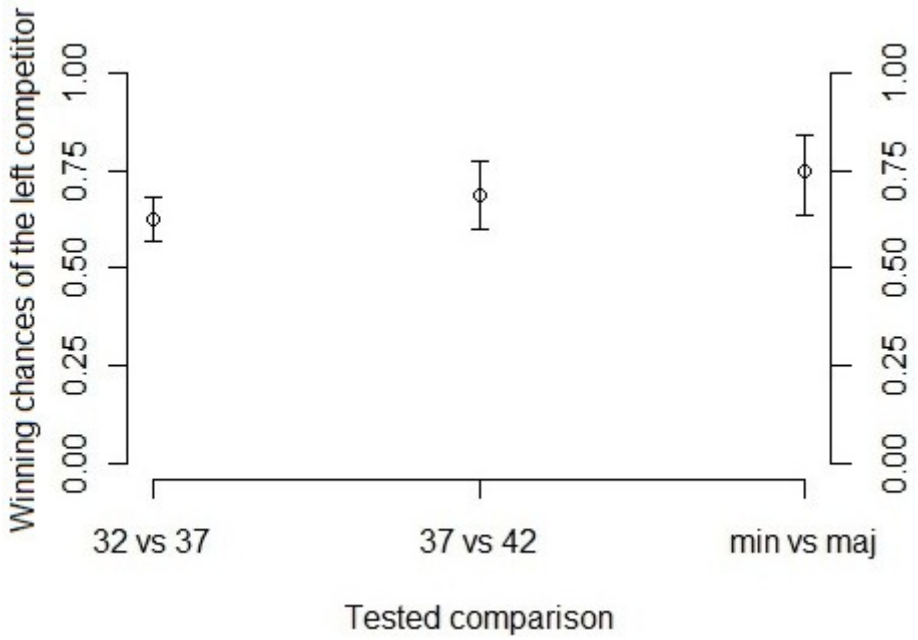

Figure 12: **Estimated winning chances of competition trial factors.** The first and second comparison show estimated winning chances for the strain evolved at the lower temperature. The third comparison shows the estimated chance of the minority out-competing the majority. The points represent the estimated chance and the bars represent 95% confidence intervals.

Discussion

Evolution experiment

The decrease of population densities was faster at higher temperatures. Thus, the first hypothesis that population densities decline faster at higher temperatures can be accepted. The reason for this temperature-dependency is probably related to increased metabolic rates at higher temperatures and increased stresses at the highest two or three temperatures (i.e. from 40°C on).

The population dynamics shown in figure 9 and figure 3 are similar and it shows how conservative and reliable *E. coli* population dynamics are.

It would be expected that the bacteria should grow to the highest densities close to their growth optimum at 37°C (Fig. 13), but the predicted curve in figure 8 has a minimum very close to 37°C. This suggests that the carrying capacities do not reflect the growth rates during exponential phase. In fact, it seems that lower growth rates during exponential phase support slightly higher carrying capacities.

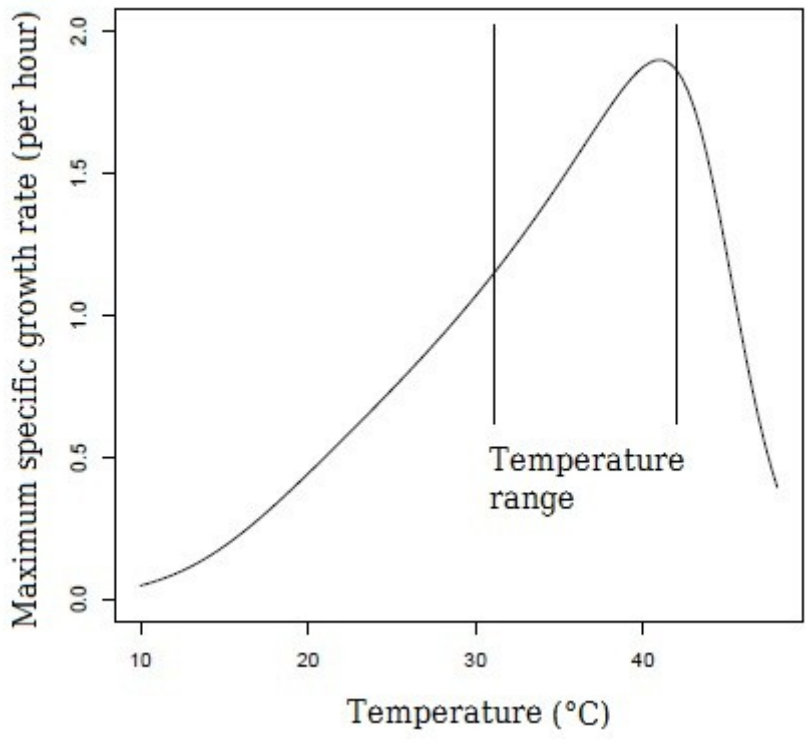

Figure 13: **Maximum specific growth rates of *E. coli*.** The response curve is based on temperature-dependent models of *E. coli* growth in exponential phase [31]. The two vertical lines show the temperature range used in the experiments. Figure adapted from previous, unpublished work.

The nalidixic acid resistant strain had significantly higher overnight densities, which means that the two strains are not neutral during the exponential growth phase. The antibiotic resistance markers of these strains have been reported to be effectively neutral when there is no drug selection [22]. But as there is evidence of the strain identity having a significant difference in the whole evolution experiment including the long-term stationary phase, the strains were not neutral in my experimental set-up. Therefore, the strain identity had to be treated as a factor in the models that try to explain the results from the competition trials.

Competition trials between weeks (compared to the ancestor)

These competition trials proved that the evolution of the GASP phenotype occurred already after two weeks or even earlier, which is in agreement with the literature [14].

The significant interactions with the temperature probably arose from the different variances of the outcomes of the three temperatures: it increased from competition trials done at 37°C to those at 32°C and was highest at 42°C. Competition trials performed at 42°C were stressful to the bacteria. In few cases neither strain formed colonies at the end of the competition trial. These more extreme outcomes explain why the variance of competition trial outcomes was so high at 42°C.

The outcomes of competition trials where the ancestor started as the minority were similar. The ancestor went extinct in many of these cases. Being in the minority can be an advantage in stationary phase, but not for ancestors that compete against an evolved population. This is because only GASP mutants are able to follow a competition strategy that allows them not to cooperate with other bacteria that try to hold up a stationary phase with no cell division [18]. Therefore, it is not surprising, that these outcomes do not vary much.

I found no effect of the evolution time on the GASP phenotype. The results indicate that the GASP phenotype, in two weeks of evolution, had saturated relative to the ancestor. That is, additional weeks of GASP evolution provided no additional GASP versus the ancestor.

A mutant in a stationary phase culture out-competed the older strain by acquiring a mutation in the lrp regulon improving the GASP ability of the mutant [27]. Thus, the populations tested against the ancestors should have considerably different mutation histories that might correlate with the evolution time. Apparently, these differences cannot be seen when one conducts competition trials only against the ancestors.

The first part of the second hypothesis that evolved populations out-compete ancestral populations can be accepted, but it could not be shown that populations with a longer evolutionary history have a higher GASP advantage.

Competition trials between temperatures

As the generalized linear model produced so many significant interactions, it is difficult to interpret the outcomes of the competition trials that were conducted between temperatures. Instead, I focused on the comparisons of single factors taking the model predictions into account.

Strains having evolved at 32°C out-compete those from 37°C, and these out-compete strains coming from 42°C. This contradicts the hypothesis that strains evolved at 37°C out-compete strains from 42°C and 32°C. *E. coli* populations have a competitive advantage when they are acclimated to their historical evolutionary temperature compared to *E. coli* populations that are not. This is even the case, when they compete at temperatures they are not acclimated to [32]. For example, at 37°C, a population acclimated to its historical evolutionary temperature of 32°C is likely to out-compete a population acclimated to 37°C with a historical evolutionary temperature different from 37°C. The strains have a historical evolutionary temperature of 37°C (personal communication with Steven E. Finkel), which would support the hypothesis, that populations from 37°C out-compete populations from the other two temperatures, because only populations from 37°C are acclimated to their historical evolutionary temperature. To conclude, it is unknown why populations from 32°C out-competed those from 37°C. It is possible that different mechanisms than GASP determined the outcomes of the competition trials and that they do not reflect evolutionary rates.

Starting as the minority significantly improved the chances of winning. This could be a result of "evolutionary cheating" where the minority manages to grow because of its rarity and its adverse effect on the growth of the majority population [18]. Some of the colony forming units were much smaller than others and it has been shown, that the rarer population has an advantage when both the small and large type are present [33]. Small colonies were not observed from the beginning and they occurred rarely, so being small was not the property of one of the strains, but rather something that evolved sometimes during the evolution experiment. This supports the observation that the minority has an advantage over the majority.

Conclusions

The GASP system provides insights about possible mechanisms through which temperature influences evolutionary rates. Populations showing a higher GASP ability have normally gone through more mutations as well.

The results indicate that the *E. coli* populations evolved more rapidly at lower temperatures which contradicts (that is if evolving the GASP phenotype is the underlying mechanism) the general understanding that higher temperatures are associated with higher metabolic rates which are directly correlated to mitochondrial and nuclear substitution rates [6].

The fastest evolution was not at the growth optimum, however the generation times are much longer in the long-term stationary phase and the starvation induces mutagenesis [21].

As the results suggest faster evolution at lower temperatures, my experiment cannot contribute to the explanation of latitudinal gradients of biodiversity in a straight forward manner. Further investigations are needed to reveal implications of these results for natural ecosystems.

Outlook

New sets of competition trials

Due to time restrictions, many possible competition trial combinations were not assessed. It would be interesting to conduct competition trials between weeks by competing populations of different evolution times that come from the same experimental line instead of competing them only against the ancestor. This could reveal patterns of increased GASP ability with evolution time.

Using more temperatures should improve the results of the competition trials between temperatures. One should also verify that neither strain is acclimated to its historical evolutionary temperature.

Sequencing

All the populations that were used in the competition trials and that had evolution times of two, five or six weeks and the two ancestor strains were prepared to be sequenced at the functional genomics center at the University of Zürich. Interesting correlations between my phenotypical studies and the underlying genomes of my cultures could be revealed by analysing the sequence data.

Acknowledgements

I would like to thank Prof. Dr. Steven E. Finkel for providing the bacterial strains and the graphs I could use from his review [15], Yves Choffat for his great help in showing the lab to me and assisting me with organizing lab tools and consumables, Dr. Aabir Banerji for help in the lab, and Roger Bachmann for proof-reading the master thesis.

Many thanks as well to our whole group and other people from the university for fruitful discussions and inputs, and to my supervisors for guiding and helping me along the course of my master thesis.

Table of figures

Literature

[1] Yasuhara, M; Hunt, G; Dowsett, HJ; Robinson, MM; Stoll, DK. **Latitudinal species diversity gradient of marine zooplankton for the last three million years.** *Ecology letters* 15 (10), 2012.

[2] Allen, AP; Brown, JH; Gillooly, JF. **Global Biodiversity, Biochemical Kinetics, and the Energetic-Equivalence Rule.** *Science* 30, 2002, 1545-1548.

[3] Fuhrman, JA; Steele, JA; Hewson, I; Schwalbach, MS; Brown, MV; Green, JL; Brown, JH. **A latitudinal diversity gradient in planktonic marine bacteria.** *PNAS* 105 (22), 2008, 7774-7778.

[4] Brown, JH; Gillooly, JF; Allen, AP; Savage, VM; West, GB. **Toward a metabolic theory of ecology.** *Ecology* 85 (7), 2004, 1771-1789.

[5] Wang, Z; Brown, JH; Tang, Z; Fang, J. **Temperature dependence, spatial scale, and tree species diversity in eastern Asia and North America.** *PNAS* 106 (32), 2009, 13388-13392 .

[6] Lourenco, JM; Glemin, S; Chiari, Y; Galtier, N. **The determinants of the molecular substitution process in turtles.** *Journal of evolutionary biology* 26 (1), 2013, 38-50.

[7] Allen, AP; Gillooly, JF; Savage, VM; Brown, JH. **Kinetic effects of temperature on rates of genetic divergence and speciation.** *PNAS* 103 (24), 2006, 9130-9135.

[8] Lumbsch, HT; Hipp, AL; Divakar, PK; Blanco, O; Crespo, A. **Accelerated evolutionary rates in tropical and oceanic parmelioid lichens (Ascomycota).** *BMC Evolutionary Biology* 8, 2008.

[9] Bargelloni, L; Ritchie, PA; Patarnello, T; Battaglia, B; Lambert, DM; Meyer, A. **Molecular Evolution at Subzero Temperatures: Mitochondrial and Nuclear Phylogenies of Fishes from Antarctica (Suborder Notothenioidei), and the Evolution of Antifreeze Glycopeptides.** *Molecular Biology and Evolution* 11 (6), 1994, 854-863.

[10] Gillooly, JF; Brown, JH; West, GB; Savage, VM; Charnov, EL. **Effects of size and temperature on metabolic rate.** *Science* 293 (5538), 2001, 2248-2251.

[11] Bronikowski, AM; Bennett, AF; Lenski, RE. **Evolutionary adaptation to temperature. VII. Effects of temperature on growth rate in natural isolates of Escherichia coli and Salmonella enterica from different thermal environments.** *Evolution* 55 (1), 2001, 33-40.

[12] Bennett, AF; Dao, KM; Lenski, RE. **Rapid evolution in response to high-temperature selection.** *Nature* 346 (6279), 1990, 79-81.

[13] Zambrano, MM; Kolter, R. **GASPing for Life in Stationary Phase.** *Cell* (86), 1996, 181-184.

[14] Zambrano, MM; Siegele, DA; Almiron, M; Tormo, A; Kolter, R. **Microbial Competition: Escherichia coli Mutants That Take Over Stationary Phase Cultures.** *Science* 259 (5102), 1993, 1757-1760.

[15] Finkel, SE. **Long-term survival during stationary phase: evolution and the GASP phenotype.** *Nature Reviews Microbiology* 4 (2), 2006, 113-120.

[16] Corzett, CH; Goodman, MF; Finkel, SE. **Competitive Fitness During Feast and Famine: How SOS DNA Polymerases Influence Physiology and Evolution in Escherichia coli.** *Genetics* 194 (2), 2013. 409-+.

[17] Vasi, FK; Lenski, RE. **Ecological strategies and fitness tradeoffs in Escherichia coli mutants adapted to prolonged starvation.** *Journal of Genetics* 78 (1), 1999, 43-49.

[18] Vulic, M; Kolter, R. **Evolutionary cheating in Escherichia coli stationary phase cultures.** *Genetics* 158 (2), 2001, 519-526.

[19] Torkelson, J; Harris, RS; Lombardo, MJ; Nagendran, J; Thulin, C; Rosenberg, SM. **Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation.** *EMBO Journal* 16 (11), 1997, 3303-3311.

[20] Foster, PL. **Adaptive mutation in Escherichia coli.** *Journal of Bacteriology* 186 (15), 2004, 4846-4852.

[21] Loewe, L; Textor, V; Scherer, S. **High deleterious genomic mutation rate in stationary phase of Escherichia coli.** *Science* 302 (5650), 2003, 1558-1560.

[22] Finkel, SE; Kolter, R. **Evolution of microbial diversity during prolonged starvation.** *PNAS* 96 (7), 1999, 4023-4027.

[23] Stevens, MHH; Sanchez, M; Lee, J; Finkel, SE. **Diversification rates increase with population size and resource concentration in an unstructured habitat.** *Genetics* 177 (4), 2007, 2243-2250.

[24] Palchevskiy, V; Finkel, SE. **Escherichia coli competence gene homologs are essential for competitive fitness and the use of DNA as a nutrient.** *Journal of Bacteriology* 188 (11), 2006, 3902-3910.

[25] Notley-McRobb, L; King, T; Ferenci, T. **rpoS mutations and loss of general stress resistance in Escherichia coli populations as a consequence of conflict between competing stress responses.** *Journal of Bacteriology* 184 (3), 2002, 806-811.

[26] Patten, CL; Kirchhof, MG; Schertzberg, MR; Morton, RA; Schellhorn, HE. **Microarray analysis of RpoS-mediated gene expression in Escherichia coli K-12.** *Molecular Genetics and Genomics* 272 (5), 2004, 580-591.

[27] Zinser, ER; Kolter, R. **Prolonged stationary-phase incubation selects for lrp mutations in Escherichia coli K-12.** *Journal of Bacteriology* 182 (15), 2000, 4361-4365.

[28] Madan, R; Kolter, R; Mahadevan, S. **Mutations that activate the silent bgl operon of Escherichia coli confer a growth advantage in stationary phase.** *Journal of Bacteriology* 187

(23), 2005, 7912-7917.

[29] Madan, R; Moorthy, S; Mahadevan, S. **Enhanced expression of the bgl operon of Escherichia coli in the stationary phase.** *FEMS Microbiology Letters* 288 (1), 2008, 131-139.

[30] R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

[31] Heitzer, A; Kohler, HPE; Reichert, P; Hamer, G. **Utility of Phenomenological Models for Describing Temperature Dependence of Bacterial Growth.** *Applied and Environmental Microbiology* 57 (9), 1991, 2656-2665.

[32] Bennett, AF; Lenski, RE. **Evolutionary adaptation to temperature .6. Phenotypic acclimation and its evolution in Escherichia coli.** *Evolution* 51 (1), 1997, 36-44.

[33] Rozen, DE; Lenski, RE. **Long-term experimental evolution in Escherichia coli. VIII. Dynamics of a balanced polymorphism.** *American Naturalist* 155 (1), 2000, 24-35.