Non transitive fitness in a long term evolution experiment with *Aspergillus nidulans*.

Master Biology Thesis (GEN-80433), Genetics

11 February 2016

Abstract - The *Aspergillus nidulans* (a filamentous fungus) long-term evolution experiment (abbreviated to A.L.E.X.) was started to investigate evolution in a simple multicellular organism. The fitness of evolved populations is usually charted through competitive fitness assays where the evolved strain is put into direct competition with the ancestral strain. In measuring fitness in this manner, the fitness is assumed to be transitive: i.e. the cumulative fitness improvement relative to the ancestor can be predicted by the incremental gains of fitness over constituent time intervals. This thesis shows that the fitness increases of two evolved strains of *A. nidulans* show non-transitivity when measured through direct competition with the ancestor (i.e. fitness increases, then decreases again), while direct competition of samples from different time points against a colour mutant of a recent predecessor shows a fitness pattern that is in accordance with known models (i.e. the measured fitness increases, then levels off). Fourteen time points out of the 52-week evolution experiment were used. The emergence of polymorphisms during evolution is proposed as a possible cause of this non-transitivity.

Mirre Klatter
Student number: 910907438090
Study: MBI
Supervisor: Arjan de Visser

Table of Contents

Preface

"Nothing in evolution makes sense except in the light of evolution"(Dobzhansky, 1973)

The quote above is a cliché, and one I have read more than once. Clichés are popular for a reason, though, and this one is no exception. Biologists have been fascinated with the concept of evolution even since before Charles Darwin introduced the idea of adaptation through natural selection in his book 'On the Origin of Species'. It is no different for me. To me the field of evolution is one of the most exciting areas of study. It is built on classic ideas that have withstood the hand of time, which can now be further studied by using both new and old techniques.

Introduction

The study of evolution is an important field within biology, and has long relied on comparative analyses of living organisms. Such studies do not allow evolutionary change or the dynamics thereof to be observed more directly. By using microorganisms in an artificially manipulated environment, researchers can investigate many generations in a short time (Elena & Lenski, 2003).

Microorganisms have several advantages over other organisms used in evolutionary research. They have a shorter generation time, are easily manipulated and kept in laboratory conditions and much is known of their genetics. One of the biggest advantages is that samples of each time period can be stored in a non-evolved state by freezing at -80°C (Elena & Lenski, 2003).

For single-celled organisms, the best-known experiment is that of Lenski (Lenski, Rose, Simpson, & Tadler, 1991). A population was established from a single clone of the bacterium *Escherichia coli* and split into 12 lines. Each line was transferred daily with a 1% bottleneck into a new flask with fresh growth medium, and samples were stored in a non-evolved state of time points in evolution. The different evolved lines could then be compared with several techniques, amongst others competitive fitness assays in which evolved lines were put in direct competition with the ancestors to determine changes in fitness in the evolutionary environment.

Compared to single celled organisms, multicellular organisms have a more complex way of living, meaning fitness will be less easily changed. The fitness of any evolutionary unit can be broken down into two basic components: reproduction and survival. In a single-celled organism, the fitness is determined by the speed with which the cell can divide. In multicellular organisms, the more complex interactions between cells, mean that improved performance (i.e. faster growth) in a single cell will not necessarily translate into an improved fitness, for example when improved performance takes away resources from other cells and processes within the organism. The fitness will not be defined by a speed of division, but by the progress through several life stages, making the consequences of adaptation more complex and less easy to predict (Elena & Lenski, 2003; Nieuwenhuis, 2006). In addition, the higher complexity of multicellular organisms allows for more complex interactions between individuals. Different variations or polymorphisms of the same strain might fill different niches in the environment, which introduces even more variables that can affect the fitness of a population. Rainey and Travisano observed that the aerobic bacterium *Pseudomonas fluorescens* evolved variant morphs that competed among the newly evolved niche-specialists when provided with the opportunity for special heterogeneity (de Visser & Lenski, 2002).

The *Aspergillus nidulans* long-term evolution experiment (abbreviated to A.L.E.X.) was started to investigate evolution in a simple multicellular organism. The fungus has the requirements for experimental evolution (Elena & Lenski, 2003): a short generation time, can be easily propagated and manipulated and can be stored in suspended animation (Swart & Debets, 2004). The fungus has a life cycle with different life-stages, which means that the fitness of the organism depends on all these stages. The evolutionary environment is seasonal, with a limited amount of resources and space that are gradually depleted between transfers. Adaptations that increase competitive ability for resources and space are expected to evolve.

A. nidulans is a filamentous fungus that forms a mycelial network from which conidiophores and cleistothecia are formed for respectively asexual and sexual reproduction. In the wild type fungus the ripe conidiospores (conidia) have a green colour. A yellow colour mutant has been selected, which makes identification of different strains in a single culture possible, e.g. in competition assays.

The golden standard for assessing the fitness of evolved strains is by direct competition with the ancestor (Elena & Lenski, 2003). In measuring fitness in this manner, the fitness is assumed to be transitive: i.e. the cumulative fitness improvement of an evolved strain relative to the ancestor can be predicted by the incremental gains of fitness over the constituent time intervals. The principle of adaptation through natural selection is based on the premise that high fitness (i.e. high number of descendants) will cause the traits that cause that high fitness to have a larger presence in subsequent generations: in other words, it is the traits that cause high fitness that are selected for throughout evolution. Since high-fitness traits are selected for, it seems counter-intuitive for fitness to decrease over evolution, as with non-transitive fitness interactions.

Even so, several studies have documented non-transitive interactions. Paquin and Adams found a decrease in fitness in asexual evolving populations of *Saccharomyces cerevisiae*, when fitness was based on competition with the ancestral strain. This decrease in fitness was not found when they put a series of isolates in competition against its immediate predecessor. The mechanism responsible for this non-transitivity was not identified, but epistatic interactions between adaptive mutations were proposed as a possible cause (Paquin & Adams, 1983). Another example of non-transitive fitness interactions was found by Rainey and Travisano between three different variants of *Pseudomonas fluorescens* that evolved in cultures that allowed for special heterogeneity. The non-transitive effects were based competition between newly-evolved niche-specialists. Each type had a competitive advantage when it was rare relative to one or both other types in the culture (de Visser & Lenski, 2002).

Materials and methods

For protocols used in all experiments see appendix A.

Strains

The starting *A. nidulans* strains for the ALEX lines were WG649 and WG650. These strains are identical, except for marker mutations (including spore colour). WG649 has wild (i.e. green) conidia, and WG650 has yellow conidia. From these strains 2 monoclonal baseline populations were derived, which will be indicated with nia/0 and ycnx/0 for the green and yellow ancestor respectively. All lines were stored in peptone-glycerol (2:1) at -80ºC. The lines can be revived for comparison with evolved lines. Conidia colour was used as a simple marker to score the composition of mixed cultures with counting plates.

Evolution experiment

The baseline strains nia/0 and ycnx/0 were each used to start 6 lines, meaning 12 lines in total. These lines are cultured in 30 ml glass bottles with 10ml solid Minimal Medium (Pontecorvo, Roper, Hemmons, MacDonald, & Bufton, 1953) with 0.1% glucose (from now on referred to as 'evolutionary medium'). The experiment was started by inoculating the bottles with 100 µl spore suspension with a density of 10^7 spores/ml. The bottles were shaken by hand to spread the inoculum over the medium, and incubated with loose caps for seven days at 37ºC in the dark. After incubation the spores were harvested by pouring 5ml saline (0.8g/l NaCl) with Tween80 (0.05%) in the bottle and shaking the bottle for 10 minutes at 200 rounds min^{-1}. 50 µl of the suspension was plated on fresh medium in a new bottle and 1 ml of the spore suspension was stored at -80ºC in peptone-glycerol. Weekly transfers were performed for 52 weeks. In order to detect cross-contamination, weekly transfers of green and yellow lines were alternated. If contamination from spores from a different strain occurs and reach frequencies of ~1% or higher in the invaded population, different coloured colonies would have been detected in controls (i.e. yellow colonies in wild type evolving strain or vice versa). No cross-contaminations were observed.

Competitive fitness assay

Against ancestor

The fitness of the evolved lines nia1 and nia3 was determined through direct competition with the ancestral lines in the evolutionary environment. Before competition, strains were first grown for a week on evolutionary medium to remove the effects of the glycerol in which they are stored, and then grown for another week to condition strains to the evolutionary environment. The spores were counted with the Coulter counter (see appendix A), and a mixture of ancestor (yellow): evolved (green) was made with a ratio of 1:1 and a spore density of $2*10^6$ spores/ml. 100 µl of this 1:1 mixture was inoculated on fresh medium in the evolutionary environment and incubated with loose caps for seven days at 37ºC in the dark.

Both before and after competition, the ratios of both competitors were determined by inoculating counting plates (complete medium with triton and 0.5% glucose) and counting green and yellow colonies, with the numbers ranging from approximately 1000-50 total colonies counted per individual measurement (full dataset can be found in the "Mirre Klatter – supplementary data.xlsx" file). With these ratios the following formula was used to calculate the selection rate constant (r) i.e. the difference in fitness of the two strains (Lenski et al., 1991):

$$r = \ln\left(\frac{\#\ of\ evolved\ colonies\ after\ competition}{\#\ of\ ancestral\ colonies\ after\ competition}\right) - \ln\left(\frac{\#\ of\ evolved\ colonies\ before\ competition}{\#\ of\ ancestral\ colonies\ before\ competition}\right)$$

Each assay was performed in triplicate for every time point (14 in total with 4-week intervals) of the two evolved populations. A visual representation of these competitions can be found in Figure 1. The fitness as measured through direct competition with the ancestor will be called 'a-fitness'.

Figure 1: A visual representation of competitions that were performed. The two evolved lines in green were competed against the yellow ancestor for each time point in the competition. There were 14 competitions per replica block. Numbers indicate weeks from the start of the evolution experiment.

Against recent predecessor

To determine whether non-transitive fitness interactions are occurring, the fitness of both evolved lines was also determined through direct competition of each time-point sample against a recent predecessor. In order to compare evolved lines with a recent predecessor, yellow-coloured mutants were selected for every other selected time point after treatment of spores with UV light. To select the strain with the least adverse effects of the mutation, a mycelium growth rate measurement (see appendix A) was used. The colour mutants were put in competition against the previous and following time points, as well as the original (i.e. with green spores) wild-type sample from the same time point as a reference. Each assay was performed in triplicate for every time point (14 in total with 4-week intervals) of the two evolved populations. A visual representation of these competitions can be found in Figure 2. The fitness as measured through direct competition with a recent predecessor, will be called 'rp-fitness'.

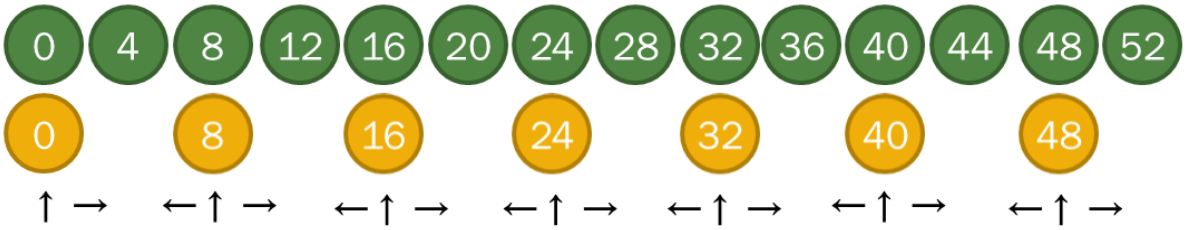

Figure 2: A visual representation of competitions that were performed. Colour mutants were selected for every other time point, which were put in competition with the time point before it (←), itself (↑, to estimate the effect of the spore-colour mutation), and the time point after it (→). There were a total of 20 competitions per replica. Numbers indicate weeks from the start of the evolution experiment.

Finding polymorphisms

Both lines were plated out for all experimental time points to find out whether polymorphisms have appeared in the evolutionary mix. The inoculations were done on evolutionary medium, to verify that the polymorphisms seen were also polymorphisms present in the evolutionary environment. Concentrations of 10^{-3}, 10^{-4} and 10^{-5} were plated, and photographed after 1 week of incubation at 37^{o}C. Photographs were taken, and colonies were counted manually with the point picker tool in imageJ. Two phenotypes were distinguished: the 'wild-type' with visible spores and low amounts of mycelium; and the 'low-spore' phenotype, which are given a white appearance by low spore counts and fluffy mycelium growth (Figure 3).

Figure 3: Picture of a plate used for phenotype quantification. Pictured is the 10-4 concentration of nia 1 week 28. The bottom three colonies show the 'low-spore count' phenotype, and the top colony shows the 'wild-type' phenotype.

Spore yield

The spore density of the spore suspensions used for the competition mixes was measured with the coulter counter. These spore densities are equivalent to the spore density during the evolutionary experiment, because the strains were first grown for a week on evolutionary medium to remove the effects of the glycerol in which they are stored, and then grown for another week to condition the strains to the evolutionary environment.

Results

Competitive fitness

Against ancestor

The two lines show a similar pattern in the fitness over time when measuring fitness through direct competition with the ancestor: there is a steep increase in fitness for the first weeks of evolution, followed by a period where the fitness levels off, and then a decline in fitness followed by another increase (Figure 4 and Figure 5). This is not what we would expect to happen with fitness if fitness improvements were caused by improvements of competition for the same limiting resource (de Visser & Lenski, 2002). A popular model for fitness increase over evolutionary time is a rectangular hyperbola where there is an initial sharp increase that levels off over time and approaches a maximum (Paquin & Adams, 1983).

In Figure 4, one such hyperbola is fitted through the first 5 points of the fitness curve to model a predicted fitness curve. It is clear the two graphs do not match up at all. The steep incline in the beginning matches, but whereas the predicted fitness continues to increase, the measured fitness decreases after week 16.

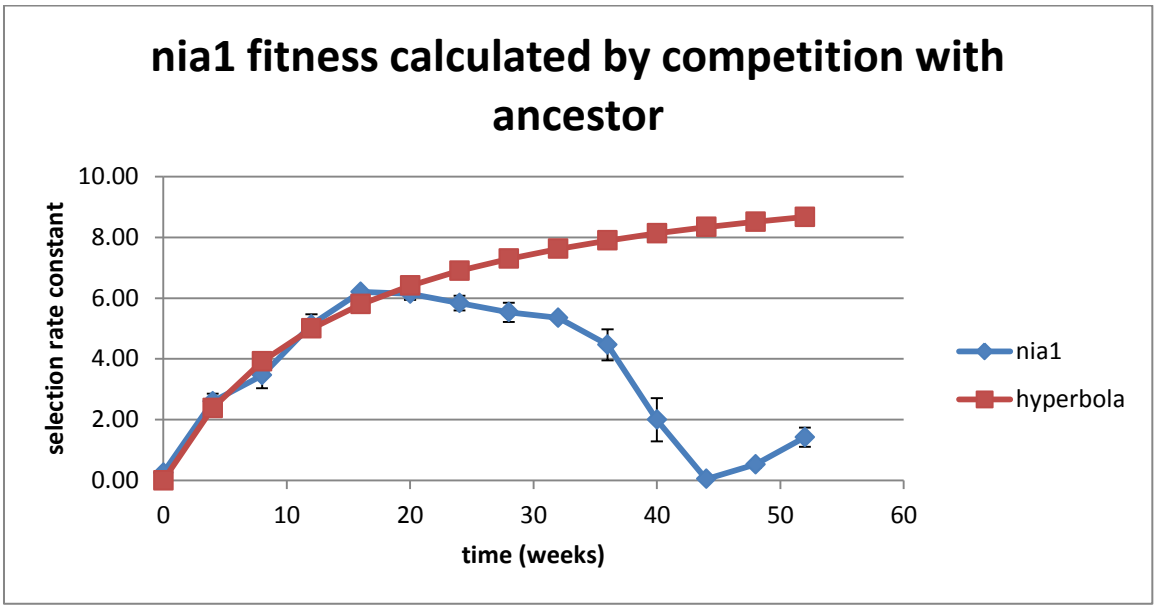

Figure 4: Comparison of fitness of population nia1 calculated through direct competition with the ancestral strain and a rectangular hyperbola fitted through the first 5 points. Each data point of the calculated fitness curve resembles the average of three biological replicate measurements. Error bars are standard error of the mean.

A comparison between the nia3 fitness curve and a hyperbola fit to the first three data points shows a similar difference. Once again the model assumes the fitness will continue to increase (albeit later slower than initially), while the measured results show decrease in measured fitness after week 12, followed by a later increase again (Figure 5).

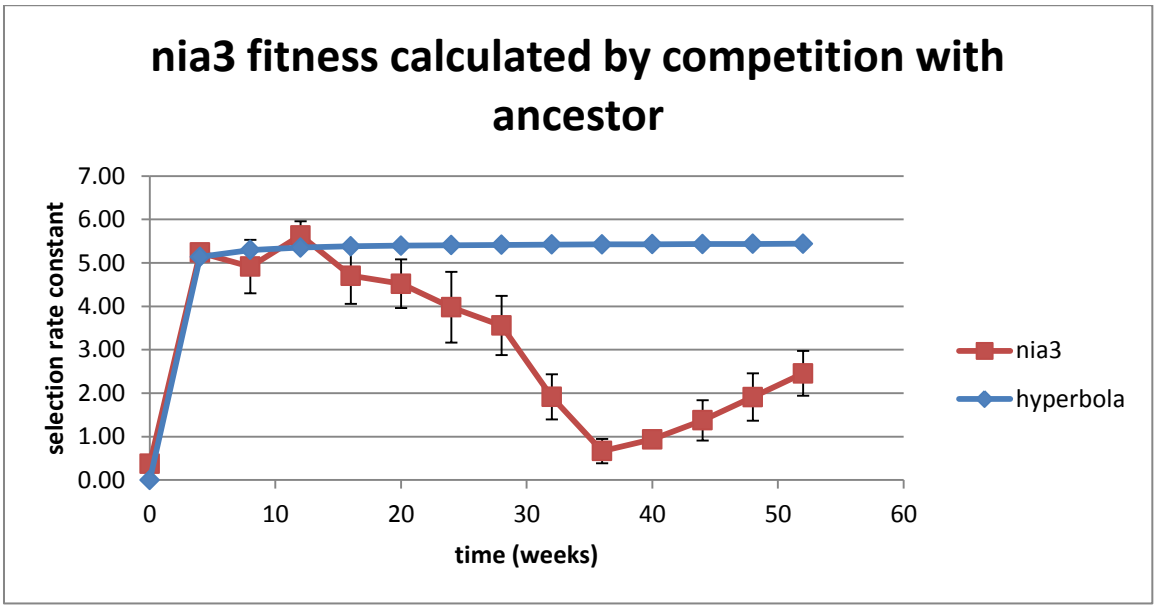

Figure 5: Comparison of fitness of population nia3 calculated through direct competition with the ancestral strain and a rectangular hyperbola fitted through the first 3 points. Each data point of the calculated fitness curve resembles the average of three biological replicate measurements. Error bars are standard error of the mean.

Against recent predecessor

The fitness curves for both strains were calculated by summing the fitness differences for all time intervals up to each time point (Figure 14 and Figure 15 in appendix B). The fitness difference between the colour mutants and original evolved populations they were picked from (Table 1) was used to correct for the mutation effect, giving the fitness curves in Figure 6 for nia1 and Figure 7 for nia3.

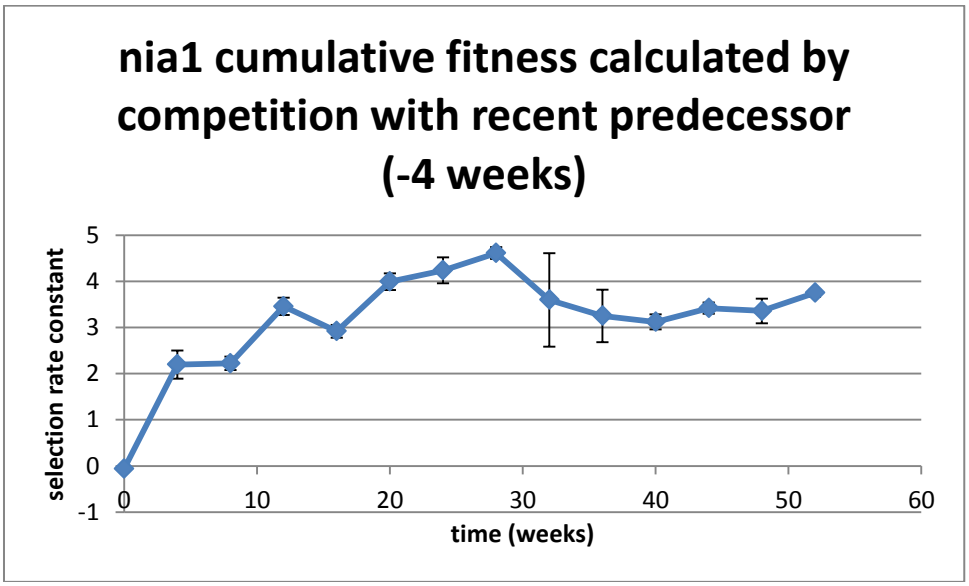

Figure 6: : Cumulative fitness of nia1 strain, obtained by calculating the sum of the fitness increments measured in competition with a recent predecessor for all preceding time intervals and corrected for fitness effects of the colour mutants. Each data point resembles the average of two biological replicate measurements. Error bars are standard error of the mean.

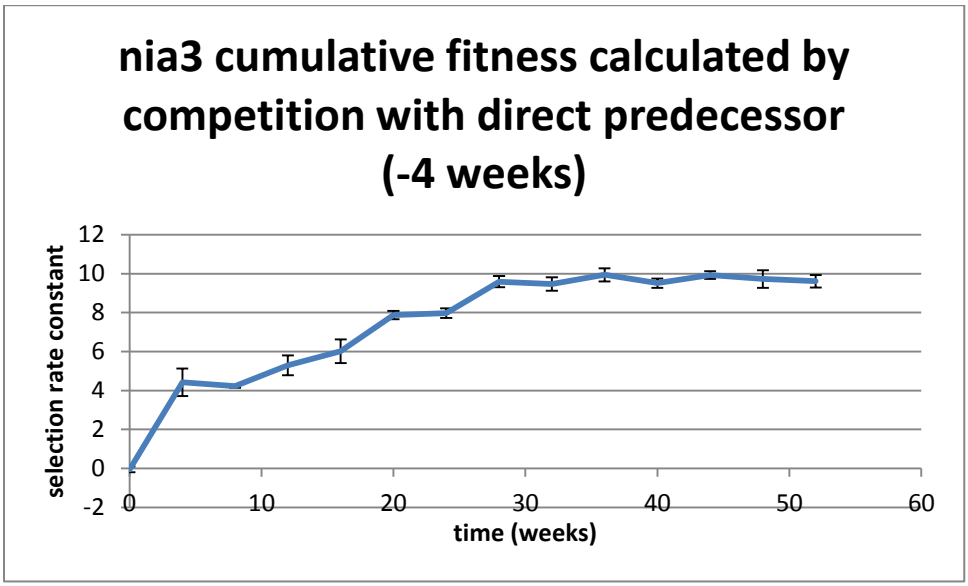

Figure 7: Cumulative fitness of nia3 strain, obtained by calculating the sum of the fitness increments measured in competition with a recent predecessor for all preceding time intervals and corrected for fitness effects of the colour mutants. Each data point resembles the average of three biological replicate measurements. Error bars are standard error of the mean.

Polymorphisms

The low-spore phenotype were absent only in earlier time points, and show a large increase in occurrence around week 28 for the nia1 strain and week 20 for the nia3 strain (Figure 8).

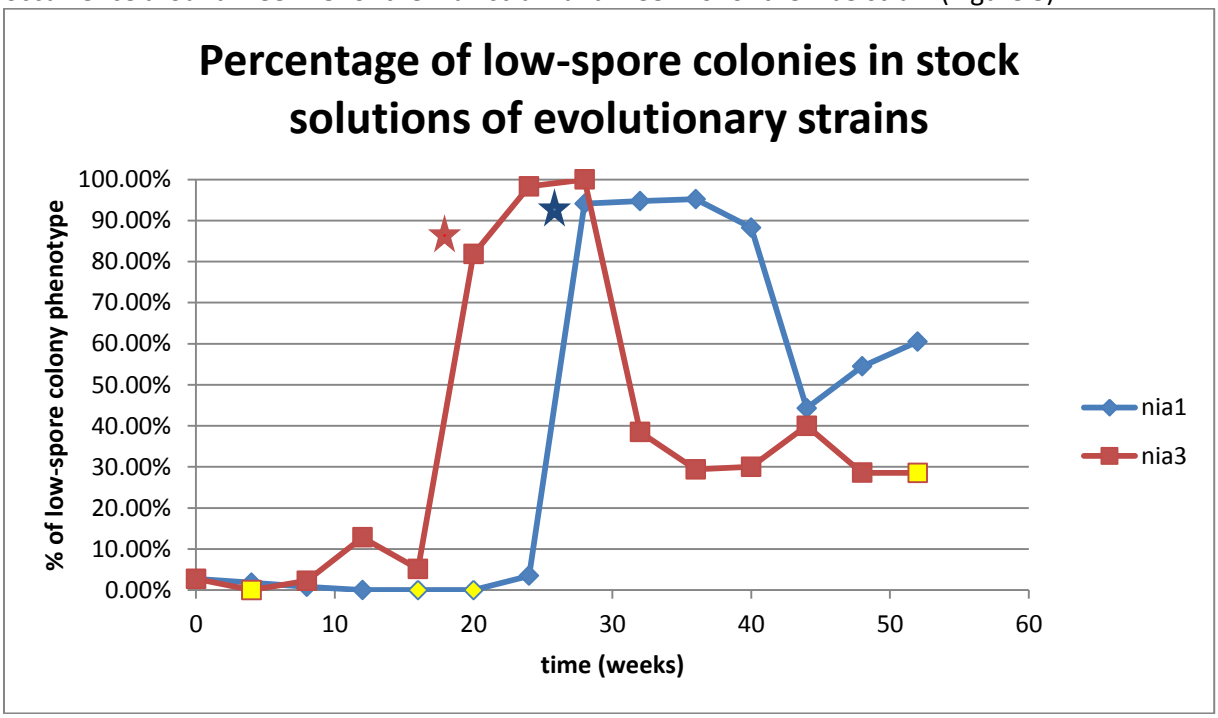

Figure 8: The frequency of low-spore colonies on MM 0.1% Glucose plates for nia1 and nia3 strains. Stars mark the time points where the percentage of low-spore colonies is over 50% for the first time. Yellow time points were calculated with <10 colonies in total.

Spore yield

The spore yield of both strains shows an initial increase, followed by a decrease (Figure 9). The time where the decrease in spore count starts, coincides approximately with the time in which a higher percentage of low-spore phenotype colonies is seen in the phenotyping experiment, i.e. week 28 for nia1 and week 20 for nia3 (Figure 8).

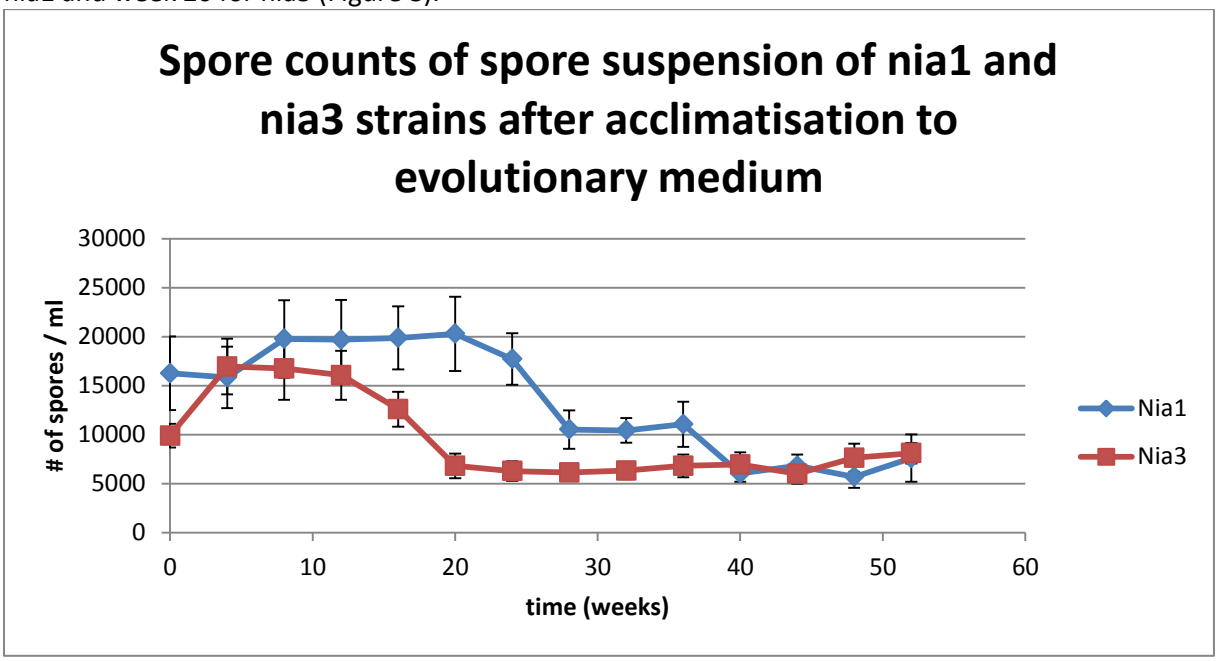

Figure 9: Spore counts of harvested spores in suspension per ml after spores from -80 ºC stock were grown for two weeks in 30ml bottles (one week for the removal of glycogen effects and one week for acclimatisation to the evolutionary medium). Spore counts initially increase during the evolutionary experiment, but drop off after week 24 for nia1 and week 16 for nia3 strains. Each data point resembles the average of six replicate measurements. Error bars are standard error of the mean.

Combined results

Since natural selection causes the traits with highest fitness to be more prevalent in subsequent generations, fitness is generally expected to be transitive, i.e. the cumulative fitness improvement of an evolved strain relative to the ancestor can be predicted by the incremental gains of fitness over the constituent time intervals. However, in populations interactions can occur that can cause non-transitive interactions to be observed, for example competition between niches (de Visser & Lenski, 2002) or so-called 'rock-paper-scissors' interactions where several types of competitors each have an advantage over each other in a cylindrical hierarchy (Kerr, Riley, Feldman, & Bohannan, 2002).

The measured fitness of the two evolved ALEX populations is different when calculated through competitions with ancestors compared with fitness calculated through competitions with recent predecessors, even though the evolved-population samples used were the same. That the measured fitness profiles are not comparable between the different methods, indicates that one or both of the results an unreliable means of measuring the change in fitness over evolution (a match between both results would have increased the confidence in the results).

A rectangular hyperbola showing a steep increase in fitness that gradually levels off, is a recognised model for the increase of fitness over evolution(Paquin & Adams, 1983). By comparing the model with the fitness curves measured by competition with the ancestor as well as competition with a direct ancestor, the method of estimating fitness that matches existing models most closely can be determined.

In the fitness curves for population nia1, the model prediction for week 52 is higher than both the a-fitness and the rp-fitness (Figure 11). Error bars do not overlap, showing that there is a significant difference between the fitness ad measured by competition with the ancestor and the recent predecessor. The rp-fitness does show a closer match with the general shape of the model, in that it levels off after the initial increase as seen in the model, rather than decrease after the initial increase as the a-fitness does (Figure 11).

The a-fitness and rp-fitness diverge almost from the start, with the a-fitness showing a stronger increase while the rp-fitness starts to level off. The decrease in a-fitness starts around the time where low-spore colonies are becoming more prevalent, as seen in the phenotyping (Figure 8) and the spore yield (Figure 9) experiments.

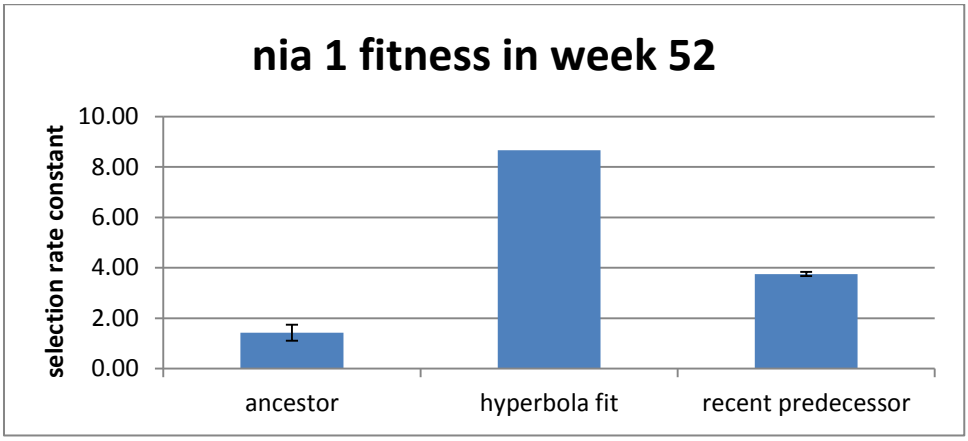

Figure 10: Fitness of population nia1 at the end of the evolution experiment (week 52) as measured by competition with the ancestor and recent predecessor, as well as a prediction obtained by fitting a rectangular hyperbola through the first 5 points. Error bars are standard error of mean.

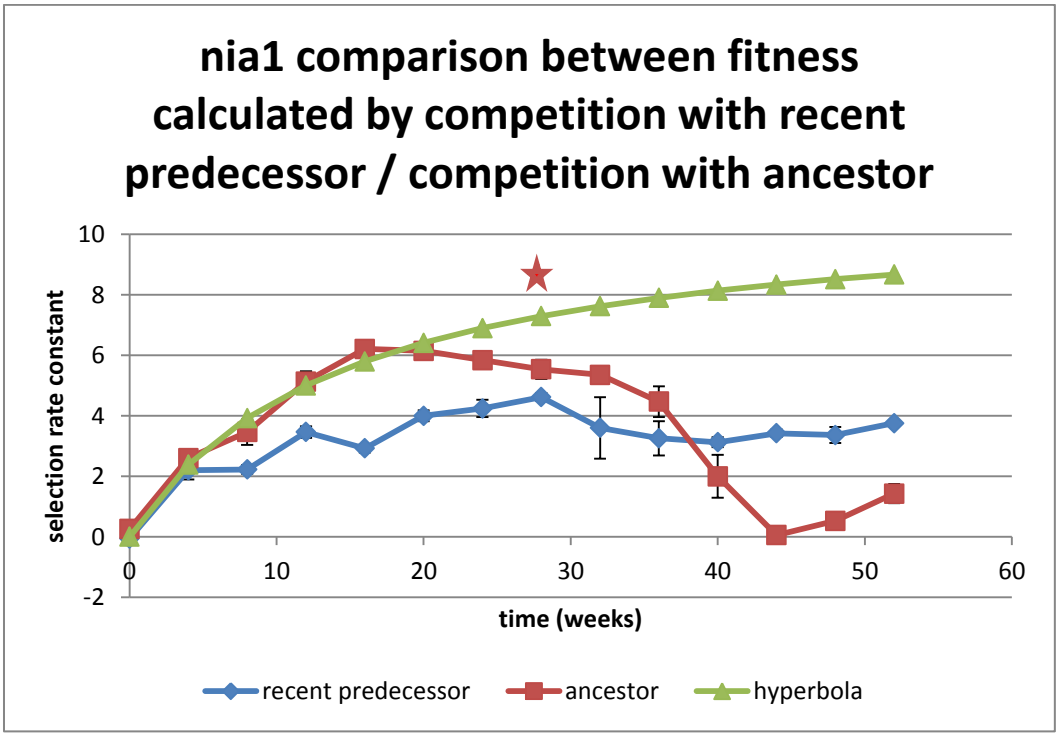

Figure 11: Fitness curves of population nia1 as measured by competition with the ancestor and recent predecessor, as well as a prediction obtained by fitting a rectangular hyperbola through the first 5 points of the competitions against the ancestor. The star marks the time point where the percentage of low-spore colonies is over 50% for the first time. Each data point of the calculated fitness curves resembles the average of three (against ancestor) or two (against recent predecessor) biological replicate measurements. Error bars are standard error of the mean.

In the fitness curves for population nia3, the model prediction for week 52 is in between the a-fitness and the rp-fitness (Figure 12). Error bars do not overlap, showing that there is a significant difference between the fitness ad measured by competition with the ancestor and the recent predecessor. The rp-fitness follows the model, in that it levels off after increasing. The initial increase of the rp-fitness continues after the model prediction has levelled off, and where the a-fitness is already decreasing (Figure 13).

The a-fitness and rp-fitness follow a similar pattern for the first four time points, after which they start to diverge. This is around the time where low-spore colonies are becoming more prevalent, as seen in the phenotyping (Figure 8) and the spore counting (Figure 9) experiments.

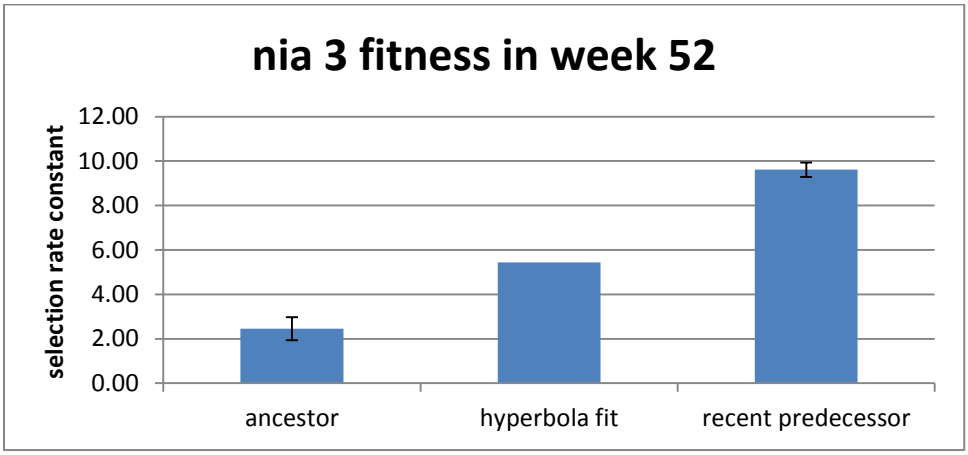

Figure 12: Fitness of population nia3 at the end of the evolution experiment (week 52) as measured by competition with the ancestor and recent predecessor, as well as a prediction obtained by fitting a rectangular hyperbola through the first 3 points. Error bars are standard error of mean.

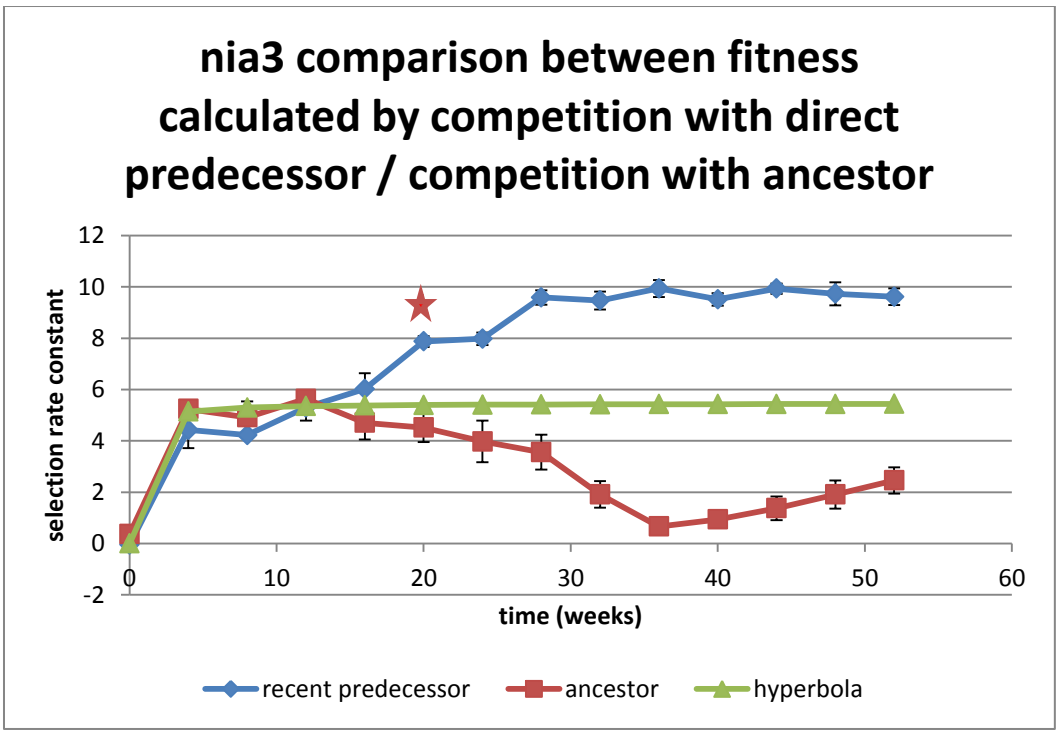

Figure 13: Fitness curves of population nia3 as measured by competition with the ancestor and recent predecessor, as well as a prediction obtained by fitting a rectangular hyperbola through the first 3 points of the competitions against the ancestor. The star marks the time point where the percentage of low-spore colonies is over 50% for the first time. Each data point of the calculated fitness curves resembles the average of three biological replicate measurements. Error bars are standard error of the mean.

Conclusion and Discussion

In both strains, the rp-fitness shows more similarities with the model prediction than the a-fitness, most importantly in that the fitness continues to increase or levels off after the initial increase rather than showing decreases. This indicates that fitness interactions are non-transitive, since the cumulative fitness thus measured is not the same as the sum of the incremental gains of fitness, as measured by competition with a recent predecessor. The divergence of the a-fitness and rp-fitness curves seems to coincide with a reduction in spore count as well as an increased prevalence of a low-spore colony phenotype. This suggests that the competitive environment has changed throughout the evolution experiment.

The low-spore colonies are very prevalent in later time periods, in spite of the low spore count that gives the phenotype its name (Figure 8). This suggests that the phenotype ensures propagation in a way other than increased spore production. Since the phenotype has a low amount of spores, this type might be a 'cheater' mutant that exploits the wild-type phenotype for spore production. Such a cheater has been found in *Neurospora crass,* where a 'somatic parasite' in a population contributes less to colony functions, but more to reproduction. The cheater thus increases its own fitness, while decreasing the colony fitness by effectively draining resources for its own reproduction (Bastiaans, Debets, & Aanen, 2015; Czaran, Hoekstra, & Aanen, 2014).

The presence of a cheater in the form of a somatic parasite would be a good explanation of the measured fitness curves. A somatic parasite reduces population fitness to increase its own fitness, which would explain why later time points (where the somatic parasite is present in the population) show a reduction in fitness in direct competition with the ancestor (a clonal population where the somatic parasite is not present). It does not fully explain why the competition with the recent predecessor shows a fitness curve that levels off, as the colour mutants used for the competition are by definition a clonal population of the high-spore phenotype (since the colour mutation can only be seen and picked in the high-spore phenotype). More research is necessary to identify the exact mechanisms that determine the difference in observed fitness between these methods of measurement.

The polymorphism screen will have to be repeated to produce more accurate data. It was done without repeats, and the low concentration of glucose in the evolutionary medium does not produce a very pronounced phenotype. Its accuracy could be improved by repeating the experiment several times on complete medium with triton for clearer distinction between phenotypes. More accurate data will make comparison with the fitness curves more reliable.

To confirm whether the low-spore count phenotype is a cheater, further experiments are necessary. Experiments that might provide additional information are frequency-dependent competitions and proximity incubations. For the first type, low-spore and wild-type isolates can be put into competition in different ratios, with the low-spore phenotype ranging from rare to abundant. If the low-spore phenotype is more successful when it is relatively rare, this indicates a dependence of fitness on the other type and hence that a cheater mechanism might be at work. For the second type of experiment, low-spore and wild-type isolates can be inoculated in proximity of each other and the resulting phenotypes and interactions (at the interphase of the two types) studied. If a yellow mutant of a wild-type isolate is used, the spore colour on the interface can show if the low-spore phenotype can somehow make use of the wild-type phenotype for spore propagation, and thus if it is a somatic parasite.

The difference in observed fitness between competition with an ancestor and competition with a recent predecessor also has implications for the use of competition with the ancestor as a standard method of determining fitness. If non-transitive fitness interactions are present, competition with the ancestor will not yield a complete picture of the changes in fitness over

evolution. Competition with colour mutants picked from recent predecessors can be used to supplement or replace fitness data gained from competitions with the ancestor.

Works cited

Bastiaans, E., Debets, A. J. M., & Aanen, D. K. (2015). Experimental demonstration of the benefits of somatic fusion and the consequences for allorecognition. *Evolution, 69*(4), 1091-1099. doi: 10.1111/evo.12626

Czaran, T., Hoekstra, R. F., & Aanen, D. K. (2014). Selection against somatic parasitism can maintain allorecognition in fungi. *Fungal Genetics and Biology, 73*, 128-137.

de Visser, J. A. G., & Lenski, R. E. (2002). Long-term experimental evolution in Escherichia coli. XI. Rejection of non-transitive interactions as cause of declining rate of adaptation. *BMC Evolutionary Biology, 2*(1), 19.

Dobzhansky, T. (1973). Nothing in biology makes sense except in the light of evolution.

Elena, S. F., & Lenski, R. E. (2003). Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nature Reviews Genetics, 4*(6), 457-469.

Kerr, B., Riley, M. A., Feldman, M. W., & Bohannan, B. J. M. (2002). Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. *418*(6894), 171-174.

Lenski, R. E., Rose, M. R., Simpson, S. C., & Tadler, S. C. (1991). Long-term experimental evolution in Escherichia coli. I. Adaptation and divergence during 2,000 generations. *American Naturalist*, 1315-1341.

Nieuwenhuis, B. P. S. (2006). Aspergillus nidulans long-term evolutionary experiment I: adaptation of simple multicellular organism to a novel environment. *MSc Thesis*.

Paquin, C. E., & Adams, J. (1983). Relative fitness can decrease in evolving asexual populations of S. cerevisiae.

Pontecorvo, G., Roper, J., Hemmons, L. M., MacDonald, K., & Bufton, A. (1953). The genetics of Aspergillus nidulans. *Advances in genetics*(5), 141-238.

Swart, K., & Debets, A. (2004). Genetics of Aspergillus *Genetics and biotechnology* (pp. 21-36): Springer.

Appendix A – Protocols

Making a spore suspension
Prepare glass tubes with 5ml of Saline-Tween (0.05% Tween) and glass beads
Pour Saline-Tween (0.05% Tween) and glass beads into the 30 ml glass bottle with the culture
Shake the bottle for 10 minutes at 200 rounds min^{-1}
Pour spore suspension in sterile 30ml glass bottle
Store at 4°C

Counting spores in suspension
Prepare the Coulter counter
Dilute 50 µl in 10 ml of Isotone II medium
Count the cells
Import in computer
Take only particles between 2.2 and 4.5 µm on computer
Total number should lie between 50,000 and 200,000

Fitness Assay
Mix equal amount of conditioned spore suspension type A with spore suspension type B, with 10E6 spores of each type (calculate in a total 400 µl volume.
Vortex
Plate 100µl of the mix on a plate (20ml of MM + 0.1% glucose)
Measure ratio before competition (see Check ratio in mix)
Incubate at 37°C for 7 days
Harvest all spores at day 7 by making a spore suspension
Measure ratio after competition (see Check ratio in mix)

Mycelium Growth Rate measurement
Incubate a plate (20 ml MM + 0.1% glucose) in the centre with a 2 µl droplet of spore suspension (E7 ml^{-1})
After 3 to 4 days measure diameter in 2 perpendicular directions
Do not move the plates around as spores might reach free medium where a new colony can start which may interfere with measurements

Picking mutants
Dilute in Saline to E-2
Plate 100µl on plate (MM + 0.5% glucose)
Expose to UV light for 10 seconds
Incubate at 37°C for 3 days
Visually check for yellow conidiophores
Pick yellow conidiophores with an incubation needle, and transfer to a fresh plate (MM + 0.1% glucose)
Incubate at 37°C for 3 days
Visually check for green conidiophores on plate
If no green conidiophores are found: harvest spores by pipetting 0.75ml saline-Tween (0.05% Tween) over the surface and storing the spore suspension 1:1 with peptone-glycerol (2:1) at -80ºC
If green conidiophores are found: repeat the picking+transfer for another purifying step

Check ratio in mix
Plate 2x50µl E-4 and 1x100µl E-4
Use plate CM + 0.5% glucose + Triton
Count total colonies after incubation at 37°C for 1 day
Count w0 colonies after incubation at 37°C for 2/3 days (when colours become visible)

Appendix B – Supplementary data

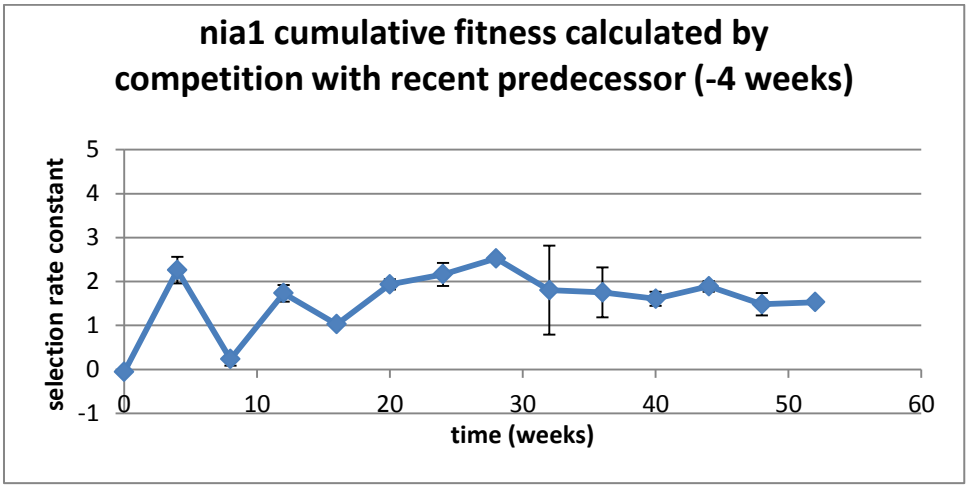

Figure 14: Raw cumulative fitness of the nia1 population, obtained by calculating the sum of the results of competition with a recent predecessor for all preceding time intervals. Each data point resembles the average of two biological replicate measurements. Error bars are standard error of the mean.

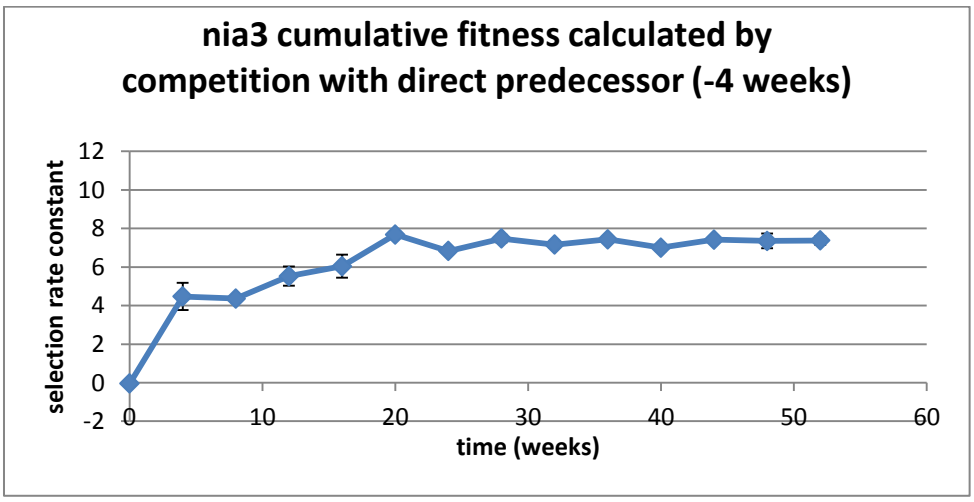

Figure 15: Raw cumulative fitness of the nia3 population, obtained by calculating the sum of the results of competition with a recent predecessor for all preceding time intervals. Each data point resembles the average of three biological replicate measurements. Error bars are standard error of the mean.

Table 1: Fitness of colour mutants compared to wild type population they were picked from

time point (weeks)	average fitness (r)	standard error of mean
0	-0.0458074	0.10596125
8	-0.0939899	0.02357043
16	0.20935661	0.1588282
24	0.96209964	0.19963648
32	0.19706051	0.29545435
40	0.00253101	0.16147347
48	-0.1347362	0.23400845