Master's Thesis

Silver-spoon effects increase competitive fitness in *Neurospora crassa*

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Tools that help organisms to adjust to environmental changes are important for their survival, as the possibility of change is constantly present in nature. Among these tools are between-generation effects, that can help in adaptation via cues from the parents. The purpose of this thesis was to investigate if between-generation effects exist in ascomycete *Neurospora crassa*, and if they do, what is their transmission mechanism and effect on offspring fitness. Between-generation effects were studied separately in relation to either sucrose concentration in the environment, or temperature. *N. crassa* was grown in either of two parental environments for two generations. In the third generation, equal amounts of spores were transferred to grow in an environment that was similar or different to the parental environment. Transmission mechanism was explored using mutant strains with deficiencies in epigenetic mechanisms, and measurements of spore size. Fitness effects were studied by competing marked strains originating from different parental environments against one another. The results show that between-generation effects exist in *N. crassa*, but the transmission mechanism for these effects is not clear. If the parents originated from environments 1.5% sucrose or 25 °C, offspring grew better irrespective of their immediate environment. Between-generation effects are also able to give offspring significant competitive advantages in laboratory conditions. Based on these results, *N. crassa* is able to benefit from between-generation effects and so these effects can be important in nature.

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Muuttuviin ympäristöoloihin sopeutumista helpottavat työkalut ovat eliöille tärkeitä, koska muutoksen mahdollisuus on luonnossa jatkuvasti läsnä. Näiden työkalujen joukkoon kuuluvat ylisukupolviset efektit, jotka voivat tarjota jälkeläisille keinoja sopeutumiseen vanhemmilta saatavien vihjeiden välityksellä. Tässä tutkielmassa selvitettiin ylisukupolvisten efektien esiintymistä *Neurospora crassa* -kotelosienellä, ja efektien löytyessä niiden siirtomekanismia ja vaikutusta jälkeläisten kilpailukykyyn. Ylisukupolvisia efektejä tutkittiin erikseen suhteessa ympäristön sakkaroosipitoisuuteen ja lämpötilaan. *N. crassa* kantoja kasvatettiin jommassakummassa kahdesta ympäristöstä kahden sukupolven ajan. Kolmannessa sukupolvessa sama määrä itiöitä jokaisesta näytteestä siirrettiin kasvamaan ympäristöön, joka oli joko samanlainen tai erilainen vanhempien ympäristön kanssa. Siirtomekanismia tutkittiin mutanttikantojen avulla, joilla oli puutteita jonkin epigeneettisen modifikaation tuottamisessa. Lisäksi itiöiden koko mitattiin. Kelpoisuusvaikutuksia selvitettiin kilpailuttamalla eri ympäristöistä peräisin olevia rihmastoja toisiaan vastaan. Tulokset osoittavat, että ylisukupolvisia efektejä esiintyy *N. crassa*lla, mutta niiden siirtomekanismi on epävarma. Jos vanhemmat tulivat 1.5% sakkaroosipitoisuudesta tai 25 °C, jälkeläiset kasvoivat paremmin niiden välittömästä ympäristöstä riippumatta. Ylisukupolviset efektit antoivat jälkeläisille myös merkittäviä kilpailullisia etuja laboratorio-olosuhteissa. Näiden tulosten perusteella *N. crassa* pystyy hyötymään ylisukupolvisista efekteistä ja siten nämä efektit voivat olla tärkeitä myös luonnossa.

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TERMS AND ABBREVIATIONS

ABBREVIATIONS

PE	parental environment
AE	assay environment
CE	competition environment

1 INTRODUCTION

1.1 Phenotypic plasticity

Phenotypic plasticity allows rapid adjustment to immediate environmental circumstances by allowing the organism to produce different phenotypes depending on their living conditions (West-Eberhard 1989). For example, plants can modify their growth based on available light conditions to gain the optimal amount of light (Bell and Galloway 2007, Baker et al. 2018), and birds can modify their egg laying date based on temperature (Nussey et al. 2005). Plasticity can also occur in response to biotic forces such as interactions between predators and prey (Tollrian and Heibl 2004) or herbivore and host plant (Agrawal et al. 2002). Evolutionary change in a trait value usually doesn't occur within one generation, as the effects of selection on current generation are often seen only in the next generation. By this time, the environmental conditions might have changed once more. Therefore, within-generation phenotypic plasticity could be useful especially in environments that change faster than what is the generation time of the organism in question.

1.2 Between-generation effects

Plasticity can also occur between generations. This form of plasticity has gone by names maternal effect (Fox et al. 1997) and transgenerational plasticity (Galloway and Etterson 2007), among others. In this type of plasticity, the parent influences the offspring phenotype through other means than direct inheritance of genes (Räsänen and Kruuk 2007, Wolf and Wade 2009). In this thesis, I call this phenomenon as between-generation effect and focus on the effect of parental environment on offspring performance. Between-generation effects are thought to be most useful for sessile organisms such as plants, as they are unable to relocate in case of environmental change (Galloway and Etterson 2007). Thus, a method for quick adaptation could be valuable. Despite between-generation effects being often

referred to as maternal effect, between-generation effects can also originate from the father (Crean et al. 2013). The parent from which these effects come from is most likely dependent on which parent's environment is a more accurate predictor of the environment the offspring will reside in, as environmental predictability is a key to understanding the development of such a system (Burgess and Marshall 2014). If the parent cannot predict the environment the offspring will face with sufficient accuracy, such anticipatory system provides no benefits to either parents or offspring.

Simple examples of a between-generation effect would be of a plant producing of larger seeds in a good quality environment (Zas et al. 2013) or larger parents producing larger eggs (Heath et al. 1999). There are also numerous more complex examples. In a study by Jobson et al. (2015) with nematode *Caenoharbditis elegans*, starvation had negative effects on the starved individuals. However, the offspring of these affected parents developed resistance to starvation and heat, though they also suffered from some decreases to fitness. These effects also lasted beyond the first generation of offspring. In another study with fall field crickets (*Gryllus pennsylvanicus*) by Storm and Lima (2010), offspring had stronger reactions to predation threat if their mothers were exposed to the predator. In plants, parental exposure to herbivory increases offspring defences (Holeski 2007, Rasmann et al. 2012). Between-generation effects have been referred to as "silver-spoon effects" in contexts where offspring benefit from good quality parents (van de Pol et al. 2006).

1.3 Transmission mechanism

1.3.1 Provisioning

Parental provisioning of food and resources to offspring is a common cause of between-generation effects, and the study mentioned above by Zas et al. (2013) related to seed size is one example of it. As provisioning is related to resources, it could be logical to assume it occurs more in good quality environments, where the parents don't need all available energy to their own growth and survival. However,

there are many examples of the opposite. In a study by Vijendravarma et al. (2010) with *Drosophila melanogaster*, malnourished parents supplied their eggs with more resources. Similar results were obtained with *C. elegans* by Harvey and Orbidans (2011). Therefore, provisioning remains as a possible source of between-generation effects even in poor environments. Provisioning would be a simple and flexible method for organisms to transmit between-generation effects, as resource benefits can be expected to benefit offspring in all kinds of environments. Therefore, it would not require a precise match between parental and offspring environments to be useful.

1.3.2 Epigenetic inheritance

Organisms can acquire epigenetic modifications throughout life. For example, this can happen through environmental influences, and the changes can later be erased (Lang-Mladek et al. 2010). Histone modification, DNA methylation and RNA interference are examples of possible epigenetic mechanisms producing these changes (reviewed by Nakao 2001, Bannister and Kouzarides 2011, Holoch and Moazed 2015). Epigenetic modifications can be used to predict binding of transcription factors to certain locations (Feng et al. 2012, Liu et al. 2015), and therefore they are linked to gene regulation. Epigenetic changes can have serious consequences for the life of an organism. For example, they have been linked to diseases such as cancer (Guo et al. 2006, Zhang et al. 2016).

In addition to being consequential for the organisms themselves, epigenetic changes can also affect the life of their offspring. Epigenetic alterations can be heritable, and between-generation effects through epigenetic inheritance have been demonstrated in multiple taxa. In addition, they can be transmitted by many different epigenetic modifications. In a study by Norouzitallab et al. (2014), water invertebrates of the genus *Artemia* were exposed to heat shocks. This led to increased resistance to heat and a pathogenic bacterium *Vibrio campbellii* both in the animals themselves and their offspring three generations onwards. These changes were transmitted most likely through inheritance of DNA methylation status and histone modifications. In

another study by Rechavi et al. (2014) with *C. elegans*, small RNAs targeted to nutrition related genes were transmitted to all three study generations after the parental generation experienced starvation. Epigenetic alterations seem to be more easily inherited between generations in plants, in comparison to e.g. mammals (reviewed by Heard and Martienssen 2014). During the production of germ cells in animals, the cells undergo epigenetic reprogramming that erases most of parental epigenetic modifications, while in plants this reprogramming machinery seems to be less stringent. As epigenetic modifications can affect progeny over multiple generations, their effects can be longer lasting than those of provisioning, which would require new investment at every generation.

1.4 Evolutionary consequences of between-generation effects

These examples show that between-generation effects (and by extension, epigenetic modifications) can have differing and long-lasting effects on organisms, perhaps over multiple generations. In many cases, between-generation effects seem to have an adaptive function in a species' ecology (e.g. Fox et al. 1997). How significant these effects are in comparison with direct environmental effects is still debatable (Uller et al. 2013), but it can be quite safely argued that potential for evolutionary role for between-generation effects exists. It has been demonstrated through mathematical models, that between-generation effects can enhance adaptation to new environments (Hoyle and Ezard 2012). This could allow organisms to persist in changing environments long enough for Darwinian evolution to modify the organism genetically. Genetic assimilation is one of the ways conversion of a trait from plastic to genetically determined could take place (Lande 2009). Genetic assimilation means, that previously plastic phenotypic trait becomes genetically set through selection on particular plastic phenotype (reviewed by Pigliucci et al. 2006). Some evidence for genetic assimilation taking place in nature exists (Diggle and Miller 2013).

Under the threat of climate change and massive environmental shifts, the study of quick adaptive mechanisms is highly important and provides further information

on the adaptive ability of different organisms. This thesis aims to discover if between-generation effects also exist in a well-known model organism, the filamentous fungus *Neurospora crassa,* in connection to different environmental conditions (namely abundance of energy source and temperature). I am aware of only one related study done previously, by Zimmerman et al. (2016). Instead of effects caused by environment, they studied parental influence on spore and perithecia production, and found support on the existence of such effects. Therefore it is plausible, that between-generation effects might exist in *N. crassa* relating to other functions as well. The study by Zimmerman et al. (2016) did not touch on the mechanism by which the between-generation effects were transmitted or how they might affect the fitness of offspring. The further aim of this study is to answer these questions and so provide completely new information on the genetic regulation and inheritance mechanisms of this species. As *N. crassa* is a widely used eukaryotic model organism, these results could possibly be expanded to other species as well. And as the relevance of between-generation effects in nature could be measured by the fitness advantages they provide, the information on how between-generation effects translate to competitive benefits could provide further information on the role of these effects on survival and species interactions.

1.5 Study questions and objectives

For this master's thesis, I have three study questions.

1. Do between-generation effects exist in *N. crassa*?
2. If between-generation effects exist, how are they transmitted to next generation?
3. Do between-generation effects provide offspring meaningful advantages against competitors?

Question (1) was studied by first growing cultures of *N. crassa* in two different environments, and then measuring growth of *N. crassa* colonies in one or the other assay environment. For question (2) I repeated the experiment for question (1), but

in addition to wild-type strain I used mutant strains with deficiencies in production of different epigenetic modifications. I also measured the size of the spores produced by the parents in the different environments. For question (3), I competed colonies originating from different parental environments against one another and measured the proportions of the competitors after one generation of competition.

2 MATERIALS AND METHODS

2.1 Study species

Neurospora crassa is an ascomycete that thrives in the tropics and subtropics after fires (Turner et al. 2001). It is a model organism and so available resources surrounding it are extensive. *N. crassa* is heterothallic, meaning that sexual reproduction can only occur between hyphae of opposite mating types *A* and *a* (Metzenberg 1979). Both mating types are able to produce reproductive structures that are analogous to those of males and females in other organisms. *N. crassa* can also reproduce asexually through spores, and switches between reproductive modes in response to environmental stimuli, such as nitrogen limitation (Ricci et al. 1991). Optimal growth conditions for *N. crassa* are 1.5% sucrose and 35 °C (Kronholm et al. 2016). After 35 °C, the growth rate starts declining rapidly (Ryan et al. 1943, Kronholm et al. 2016), indicating temperature stress.

2.2 Existence of between-generation effects

The existence of between-generation effects was tested separately in relation to two environmental factors; sucrose concentration (abundance of energy source) and temperature. Henceforth, I refer to these sub experiments as sucrose experiment 1 and temperature experiment 1, respectively. The strain used in these experiments was the commonly used wild-type strain FGSC# 2489, acquired from the Fungal Genetics Stock Center (McCluskey et al. 2010). The growth medium was Vogel's medium (Metzenberg 2003), with 1.5% agar. In the sucrose experiment, the sucrose

concentration of the medium depended on the environment (1.5% or 0.015%). In the temperature experiment, sucrose concentration was always 1.5%.

Both experiments included three generations in total. The experimental design is shown in Figure 1. For the first two generations, the samples were grown in either of the two environments. In the sucrose experiment 1, these environments were 1.5% or 0.015% sucrose in growth medium. In the temperature experiment 1, the environments were 25 °C and 40 °C. In the third generation, samples from both environments were grown in these same (parental) environments and also in the opposite environment. This design made it possible to observe growth of samples from different parental environments within the same assay environment.

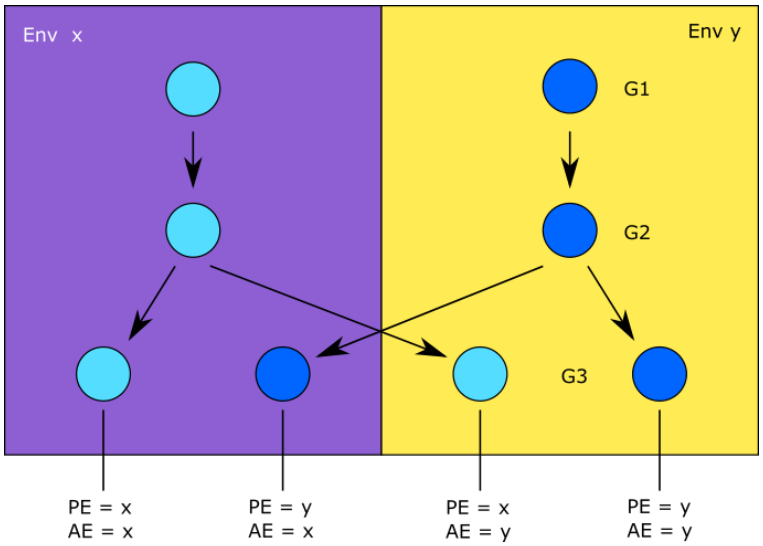

Figure 1. Spore transfer process between two environments, x and y. PE = parental environment and AE = assay environment. Samples grown in parental environment for two generations. In third generation, spores were grown either in the same environment as the previous generations, or in the opposite environment.

Seven replicates were used in all three generations. After maturation, the spores produced by the second generation were mixed with 0.01% Tween-80 solution. The spore size and concentration were measured with CASY cell counter, capillary size 45 µm and gating-window of 2.5–10 µm. Based on these concentration results, spore dilutions that contained 5000 spores μl^{-1} were made. 2 µl of these dilutions were pipetted in the middle of small petri dishes. The petri dishes were placed in a growth chamber (Hi-Point MT-313) and covered with loose

cardboard boxes to prevent the medium from drying. In the sucrose experiment 1, all samples were grown in 25 °C and constant darkness. In the temperature experiment 1, the growth conditions were the temperatures under investigation (25 °C and 40 °C) and constant darkness.

The cardboard box used to prevent drying of the samples in G3 lowered the temperature in 40 °C treatment. From the inoculation to the first growth measurement, the temperature of the samples was approximately 38 °C. Between adjacent growth measurements, the temperature of the samples rose only to approximately 36 °C. Because of this, the assay environment for temperature is hereafter referred to as 37 °C, the mean of these two values. This change in temperature should not have an effect on the outcome of the experiment in terms of parental effect however, because the environment experienced by the parental generation was 40 °C. The temperature inside the cardboard boxes was not measured for every experiment done for this thesis, but was assumed to be identical. The effect of the cardboard boxes on the 25 °C was smaller than for 40 °C, and is not considered to have an effect.

The diameter of the colony on the petri dishes was measured first time after approximately 18 hours after inoculation, and then in 4-hour intervals until the colony had reached the end of the petri dish or four growth measurements had been acquired. The size was measured by marking the largest diameter of the colony on the bottom of the petri dish at each measurement time point. The diameter was then measured by using a ruler, with ± 0.5 mm accuracy. Samples were mixed inside the cardboard boxes at the end of each growth measurement, so that the same samples were not always at the same place inside the boxes.

2.3 Transmission of between-generation effects

2.3.1 Epigenetic inheritance

To study the transmission of between-generation effects through epigenetic modifications, multiple mutant strains of *N. crassa* were used. These strains were

aof2, *dim-2*, *elp3*, *dcl-1 dcl-2*, *qde-2*, *set-1*, *set-2*, *set-7*, *nst*-1 and *hda-1*. These mutant strains have deficiencies in different epigenetic mechanisms (Kronholm et al. 2016), and so usage of these mutants allowed me to see if any of these epigenetic mechanisms was indicated as the transmission mechanism of the between-generation effects. For example, if one mutant strain produced differing results to others in terms of the between-generation effect, it could be inferred that the deficient epigenetic mechanism in this mutant works as the transmitter of the between-generation effect. The mutant strains have been backcrossed with the wild-type strain FGSC# 2489 five times, so they have identical genetic background with the wild-type strain, excluding the mutation (Kronholm et al. 2016). The strains and mutations they exhibit are listed in Table 1 (made after Kronholm et al. 2016 and Kronholm and Ketola 2018, with some modifications). This experiment with the mutants was also done separately for both sucrose and temperature, and almost identically to the one described in section 2.1. It differed only in two points. First, for practical reasons, it was impossible to maintain seven replicates through the whole experiment. Instead, three replicates of each strain were used in the first two generations (G1 and G2). In the third generation, these three replicates were combined into a single sample before measurement with the cell counter. In the plating phase, seven replicate plates were made of each sample. Only strains *dcl-1 dcl-2* and *nst-1* in the sub experiment for temperature were studied with seven biological replicates. This run also included the wild-type as a control, also with seven biological replicates. Second, the spore and 0.01% Tween-80 mixtures made after G2 were filtered through filter pipet tips (small piece of Thermolam Plus fabric placed inside a pipet tip, Metzenberg 1989) before measurement with the cell counter. This would have been useful practise also in the two other parts of this study (existence and competition) to remove hyphae from the mixtures, but the idea for the addition of this step came only after those experiments were completed. The time between spore inoculation to petri dishes and the first growth measurement was also somewhat shorter than for sucrose experiment 1 and temperature experiment 1, approximately 14 hours.

Hereafter, I will refer to the different mutant sub experiments as follows: all mutant strains and wild-type control (sucrose) = sucrose experiment 2, mutant strains *aof2, dim-2, elp3, qde-2, set-1, set-2, set-7, had-1* and wild-type control (temperature) = temperature experiment 2, mutant strains *dcl-1 dcl-2, nst-1* and wild-type control with seven biological replicates = temperature experiment 3.

Table 1. Mutant strains used to study the transmission of between-generation effects through epigenetic modifications. Strain name is the same as that of the mutated gene.

Strain	**Mating type**	**Deficient function**
aof2	A	Histone demethylase
dim-2	A	DNA methyltransferase
nst-1	A	Histone modification H4AcK16
qde-2	A	Processing of RNA resembling microRNA
set-1	A	Histone modification H3K4me3
set-2	A	Histone modification H3K36me
set-7	A	Histone modification H3K27me3
elp3	A	Histone acetyl transferase
dcl-1 dcl-2	A	Maturation of RNA resembling microRNA
hda-1	A	Deacetylation of histone H2B

2.3.2 Provisioning

To study provisioning as the cause of between-generation effects, spore size was measured with the CASY cell counter. However, because of the need to reduce the number of replicates in the mutant strain experiments, spore size data was only available from sucrose experiment 1, temperature experiment 1, and temperature experiment 3.

2.4 Competition experiment

The fitness consequences of the between-generation effects were studied by competing colonies with different parental environments against each other. The experiment was done following the protocol of Kronholm et al. (2020), using the same sucrose environments as above (1.5% and 0.015%). The strains used in this experiment were 2489 *mat A*, 2489 *mat A csr**, 2489 *mat a*, 2489 *mat a csr**. The *csr**-tag allowed the identification of spores produced by each competitor, as it is impossible to do visually. The *csr**-tag has been produced by substituting the original *csr*-sequence with a new one, which contains a barcode (Kronholm et al. 2020). The relative abundance of this marked strain was measured in each sample with HRM-PCR.

Three replicates of each sample were grown in each sucrose concentration for two generations in 25 °C and constant darkness, until spores made by the second generation could be collected. The spores from the three replicates were then combined into a single sample. Spore concentration in these samples was measured with the CASY cell counter, and dilutions that contained equal amounts of spores were made (5×10^6 ml^{-1}). The strains were paired for competitions according to Table 2, and stock solutions containing 100 µl of each competing strain were made (thus each stock solution contained 5×10^6 spores ml^{-1} in total, 2.5×10^6 ml^{-1} per participant). Strains with the same mating type were not paired, as hyphae with same mating type can fuse together and so cause confounding results (Metzenberg and Glass 1990). 2 µl of each stock combination was pipetted into test tubes with each sucrose environment and grown in 25 °C and constant darkness until spores could be collected. These spores were suspended in 1000 µl of water with 0.01% Tween-80, and 40 µl of the suspension was placed on a PCR-plate, with 10 µl 50 mM Tris (pH 8) and 0.5 M EDTA in relation 100:2. The DNA extraction was done by boiling these samples in a PCR machine (C1000 Thermal Cycler C1000a) for 10 minutes in 98 °C. The samples were frozen after extraction.

Table 2. Competing strain pairs. PE = parental environment. CE = competition environment.

Strain 1.	**Strain 1 PE**	**Strain 2.**	**Strain 2 PE**	**CE**
2489 *csr* mat A*	1.5%	2489 *mat a*	1.5%	1.5%
2489 *csr* mat A*	1.5%	2489 *mat a*	0.015%	1.5%
2489 *csr* mat a*	1.5%	2489 *mat A*	0.015%	1.5%
2489 *mat A*	1.5%	2489 *csr* mat a*	0.015%	1.5%
2489 *mat a*	1.5%	2489 *csr* mat A*	0.015%	1.5%
2489 *csr* mat a*	1.5%	2489 *mat A*	1.5%	1.5%
2489 *csr* mat A*	0.015%	2489 *mat a*	0.015%	0.015%
2489 *csr* mat A*	1.5%	2489 *mat a*	0.015%	0.015%
2489 *csr* mat a*	1.5%	2489 *mat A*	0.015%	0.015%
2489 *mat A*	1.5%	2489 *csr* mat a*	0.015%	0.015%
2489 *mat a*	1.5%	2489 *csr* mat A*	0.015%	0.015%
2489 *csr* mat a*	0.015%	2489 *mat A*	0.015%	0.015%

2.4.1 HRM-PCR

Each HRM-PCR reaction contained 5 µl of precision melt supermix, 0.2 µl of F-primer, 0.2 µl of R-primer, 2.6 µl of water and 2 µl of sample DNA from the competitions. In addition, samples with known amount of csr*-tagged strain were included for the estimation of the standard curve. HRM-PCR program used is described in Table 3. The melting curve was measured with the last three steps. The relative abundance of csr*-tagged strain in the sample could be estimated with the melting curve.

Table 3. HRM-PCR program.

Step	Temperature (°C)	Time
1.	95	2 min
2.	95	10 s
3.	60	30 s
4.	72	30 s
5.	return to step 2 (x39)	
6.	95	30 s
7.	70	1 min
8.	70 + 0.1 until 90	5 sT^{-1}

2.5 Statistical analysis

2.5.1 Existence of between-generation effects

The data from the sucrose experiment 1 and temperature experiment 1 was analysed with a linear model, with colony size at the time of second growth measurement as the dependent variable. This time point was chosen because it contained data from all samples (unlike time points 3 or 4). It also provided some robustness to the growth estimation in comparison with time point 1, as the slow growth of some samples made the growth difficult to measure. The colony size was assumed to be normally distributed. The explanatory variables were parental environment, assay environment and the interaction between them. The formula for this model was

$$y = \alpha + \beta_1 P + \beta_2 A + \beta_3 AP,$$

where y = colony size, P = parental environment, A = assay environment, α = intercept, β_1 = effect of parental environment, β_2 = effect of assay environment and β_3 = effect of interaction between parental and assay environment. The analysis

was done using RStudio, version 3.6.1. Wild-type strain in parental environment 1.5% and assay environment 1.5% was set as intercept for sucrose experiment 1 data, and wild-type in parental environment 25 °C and assay environment 25 °C for temperature experiment 1 data. The sucrose and temperature data were analysed separately.

2.5.2 Transmission of between-generation effects

The data from sucrose experiment 2 and temperature experiment 2 was analysed with an analysis of variance and then with the Tukey test for honest significant differences. Dependent variable was colony size at time point 2, and the explanatory variables were strain, parental environment, assay environment and all possible interactions between these three variables. In the sucrose experiment, the wild-type had double the amount of samples compared to the other strains. This should not have very much impact on the results, however, as the effect of parental environment was compared within samples of each strain. The data from temperature experiment 3 was analysed separately, with the same analysis of variance and Tukey tests and dependent and explanatory variables as above for sucrose experiment 2 and temperature experiment 2.

Spore size in the sucrose experiment 1 was analysed with a linear model, where parental environment was the only explanatory variable. Parental environment 1.5% and assay environment 1.5% were set as intercept. Spore size data from the temperature experiments 1 and 3 were combined and analysed with an analysis of variance and then with the Tukey test for honest significant differences. Here, the explanatory variables were strain, parental environment and the interaction between these two. Because of the combination of data, this analysis also included more wild-type samples than samples of other strains, but here also the effect of parental environment was compared within each strain.

As an alternative, I also analysed the data from temperature experiments 1 and 3 separately. Here, I analysed the data from temperature experiment 1 with the same linear model used for sucrose experiment 1, and the data from temperature

experiment 3 with the analysis of variance and Tukey test for honest significant differences (the same dependent and explanatory variables as for the combination data).

2.5.3 Competition experiment

The data from the competition experiment was analysed with Bayesian statistics. The analysis was done with RStudio (version 3.6.1.), and the packages Rstan (Carpenter et al. 2017) and Rethinking (McElreath 2015). The analysis was done following (Kronholm et al. 2020). The model used to calculate the standard curve is the same used in that study, but the MCMC estimation was run here with two chains and 3000 iterations in total, of which warmup period was 1000. The curve is supplied in Appendix 2, Figure 10. The model for competitive fitness estimation is slightly modified from the one used by (Kronholm et al. 2020). The model is:

$$x_{est,i} \sim \mathrm{N}(\mu_i, \sigma),$$

$$\log\left(\frac{\mu_i}{1-\mu_i}\right) = \alpha_{comp[i]} + (\beta_{csr} + \beta_{matA} \times m_i + \beta_P \times p_i) \times t_i,$$

$$x_{obs,i} \sim \mathrm{N}(x_{est,i}, x_{sd,i}),$$

$$\alpha_{comp[i]} \sim \mathrm{N}(0, 0.065),$$

$$\beta_{csr}, \beta_{matA} \sim \mathrm{N}(0,1),$$

$$\sigma \sim \mathrm{hC}(0,2),$$

where $x_{est,i}$ = the estimated (true) proportion of marked strain, $x_{obs,i}$ = the observed proportion of marked strain based on standard curve, $x_{sd,i}$ = error in the observed proportion of the marked strain, $a_{comp[i]}$ = the intercept for the competitions, β_{csr} = the effect of the csr*-tag, β_{matA} = the effect of mating type *A*, β_P = the effect of parental environment, t_i = number of generations the competition lasted (now one), m_i = mating type indicator (when marked strain is *mat A*, m_i = 1 and when marked strain is *mat a*, m_i = -1) and p_i = parental environment indicator (when marked strain is from 1.5%, p_i = 1, when marked strain is from 0.015%, p_i = -1, and when marked

and unmarked strains come from same parental environment, p_i = 0). The model accounts for the uncertainty in the amount of csr*-tagged strain through the use of term $x_{sd,i}$, which is the error in $x_{obs,i}$ in relation to the true amount of marked strain ($x_{est,i}$). The MCMC estimation was run with two chains for 5000 iterations in total, of which warmup period was 1000. The model converged, with $\hat{R}$ = 1.

3 RESULTS

3.1 Existence of between-generation effects

Both parental and assay environment have statistically significant effects on colony size in both sucrose experiment 1 and temperature experiment 1 (Table 4 and Figure 2). If the parental generation came from 1.5% or 25 °C, the offspring did better regardless of the assay environment.

Table 4. Statistical results of the effect of parental environment, assay environment and their interaction on colony size in sucrose experiment 1 and temperature experiment 1. PE = parental environment. AE = assay environment.

Coefficients	Estimate	Standard error	t-value	p-value
Sucrose				
Intercept	33.71	1.14	29.57	< 0.001
PE 0.015%	-10.79	1.61	-6.69	< 0.001
AE 0.015%	-10.79	1.61	-6.69	< 0.001
PE 0.015% × AE 0.015%	4.36	2.28	1.91	0.068
Temperature				
Intercept	30.57	1.45	21.09	< 0.001
PE 40 °C	-12.93	2.05	-6.31	< 0.001
AE 37 °C	13.60	2.13	6.37	< 0.001
PE 40 °C × AE 37 °C	1.05	2.96	0.35	0.727

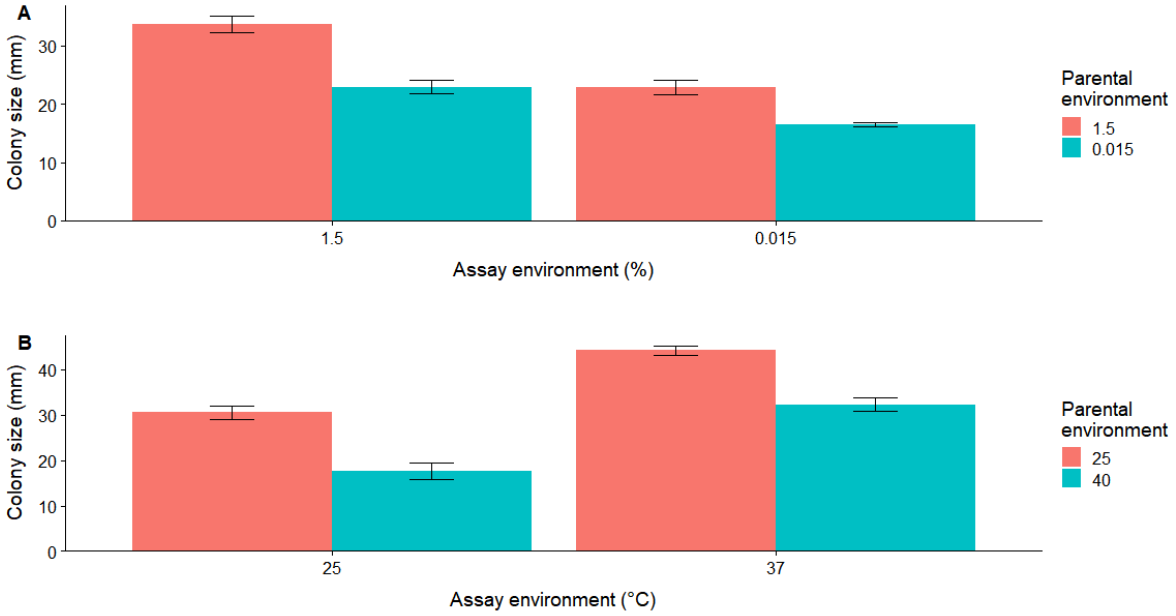

Figure 2. A) Colony size in sucrose experiment 1. B) Colony size in temperature experiment 1. Error bars represent standard error of the mean.

3.2 Transmission of between-generation effects

3.2.1 Epigenetic inheritance

In sucrose experiment 2, strains *wt, aof2, dim-2, elp3, nst-1, qde-2, set-1 and set-7* showed a statistically significant difference between the parental environments, irrespective of the assay environment (Table 5 and Figure 3). If the parents came from 1.5% sucrose, the offspring grew faster in both assay environments. Strain *dcl-1 dcl-2* had a statistically significant difference between the parental environments only in assay environment 1.5%. In this environment, the offspring grew faster if parents came from the environment with higher sucrose concentration. Strains *hda-1* and *set-2* had no statistically significant differences between the parental environments in either assay environment. ANOVA table is supplied in Appendix 1, Table 10.

Table 5. Statistical results of the effect of parental environment on colony size for sucrose experiment 2. Difference shows the effect of parental environment on colony size, when the former parental environment is compared to the latter.

Strain	Compared parental environments	Assay environment	Difference	Tukey p
wt	0.015% and 1.5%	1.5%	-5.46	< 0.001
wt	0.015% and 1.5%	0.015%	-3.07	< 0.001
aof2	0.015% and 1.5%	1.5%	-3.93	< 0.001
aof2	0.015% and 1.5%	0.015%	-4.36	< 0.001
dcl-1 dcl2	0.015% and 1.5%	1.5%	-3.07	< 0.001
dcl-1 dcl-2	0.015% and 1.5%	0.015%	-2.14	0.097
dim-2	0.015% and 1.5%	1.5%	-4.14	< 0.001
dim-2	0.015% and 1.5%	0.015%	-3.29	< 0.001
elp3	0.015% and 1.5%	1.5%	-5.29	< 0.001
elp3	0.015% and 1.5%	0.015%	-3.57	< 0.001
hda-1	0.015% and 1.5%	1.5%	-2.07	0.140
hda-1	0.015% and 1.5%	0.015%	-2.07	0.140
nst-1	0.015% and 1.5%	1.5%	-6.29	< 0.001
nst-1	0.015% and 1.5%	0.015%	-3.93	< 0.001
qde-2	0.015% and 1.5%	1.5%	-4.29	< 0.001
qde-2	0.015% and 1.5%	0.015%	-4.36	< 0.001
set-1	0.015% and 1.5%	1.5%	-3.64	< 0.001
set-1	0.015% and 1.5%	0.015%	-2.43	0.017
set-2	0.015% and 1.5%	1.5%	1.50	0.841
set-2	0.015% and 1.5%	0.015%	1.79	0.451
set-7	0.015% and 1.5%	1.5%	-3.64	< 0.001
set-7	0.015% and 1.5%	0.015%	-2.57	0.007

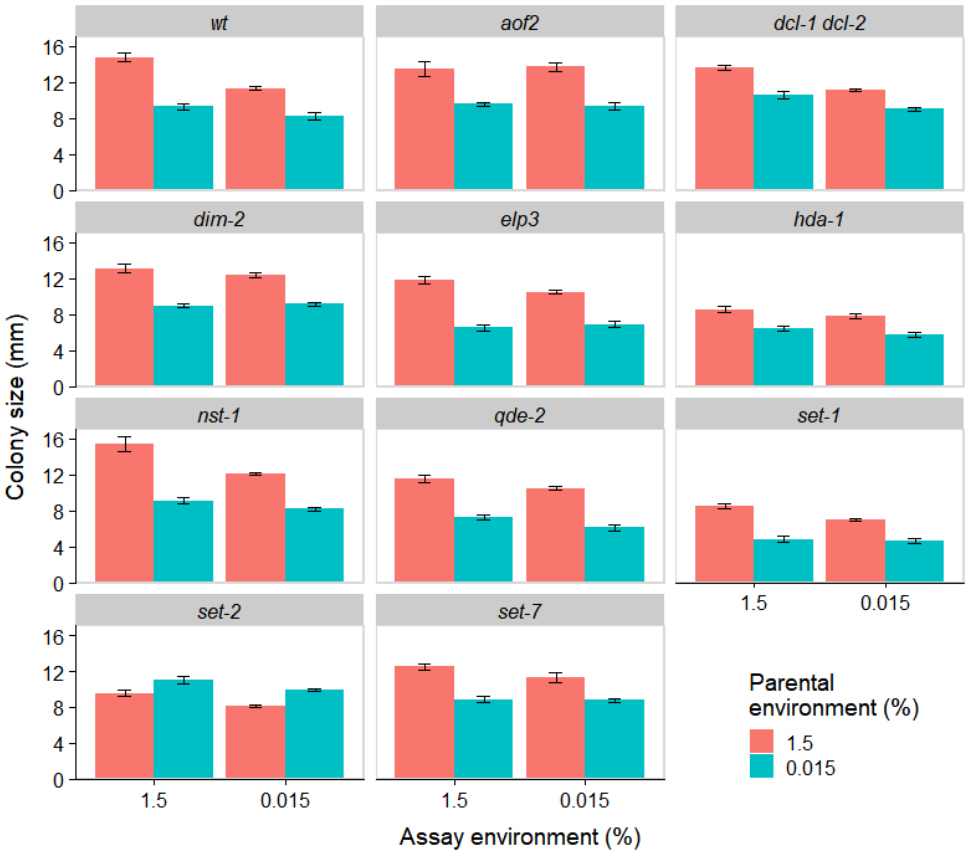

Figure 3. Colony size for strains in sucrose experiment 2. Error bars represent standard error of the mean.

In temperature experiment 2, strains *wt*, *aof2*, *hda-1*, *set-1* and *set-7* showed a statistically significant difference between the parental environments in both assay environments (Table 5 and Figure 4). If the parents came from 25 °C, the offspring grew better irrespective of their immediate environment. Strains *dim-2* and *elp3* had a statistically significant difference between the parental environments only in assay environment 40 °C. In this environment, the offspring grew better if the parents came from 25 °C. Strain *qde-2* had no statistically significant difference in parental environments in either assay environment. Strain *set-2* produced no results, as it didn't grow at all if the parents came from 40 °C. ANOVA table is supplied in Appendix 1, Table 11.

In temperature experiment 3, only strain *nst-1* had a statistically significant difference between the parental environments in both assay environments. If the parents came from 25 °C, the offspring grew better. Strains *wt* and *dcl-1 dcl-2* had no

statistically significant difference between parental environments in either assay environment. ANOVA table is supplied in Appendix 1, Table 12.

Table 6. Statistical results of the effect of parental environment on colony size in temperature experiment 2. Difference shows the effect of parental environment on colony size, when the former parental environment is compared to the latter.

Strain	**Compared parental environments**	**Assay environment**	**Difference**	**Tukey p**
wt	40 °C and 25 °C	25 °C	-12.43	< 0.001
wt	40 °C and 25 °C	37 °C	-15.05	< 0.001
aof2	40 °C and 25 °C	25 °C	-10.50	< 0.001
aof2	40 °C and 25 °C	37 °C	-17.57	< 0.001
dim-2	40 °C and 25 °C	25 °C	-4.21	0.052
dim-2	40 °C and 25 °C	37 °C	-11.43	< 0.001
elp3	40 °C and 25 °C	25 °C	-1.64	1.00
elp3	40 °C and 25 °C	37 °C	-7.50	< 0.001
hda-1	40 °C and 25 °C	25 °C	-8.00	< 0.001
hda-1	40 °C and 25 °C	37 °C	-8.00	< 0.001
qde-2	40 °C and 25 °C	25 °C	-1.43	1.00
qde-2	40 °C and 25 °C	37 °C	-3.79	0.165
set-1	40 °C and 25 °C	25 °C	-5.36	< 0.001
set-1	40 °C and 25 °C	37 °C	-14.43	< 0.001
set-2	40 °C and 25 °C	25 °C	NA	NA
set-2	40 °C and 25 °C	37 °C	NA	NA
set-7	40 °C and 25 °C	25 °C	-5.07	0.003
set-7	40 °C and 25 °C	37 °C	-10.00	< 0.001

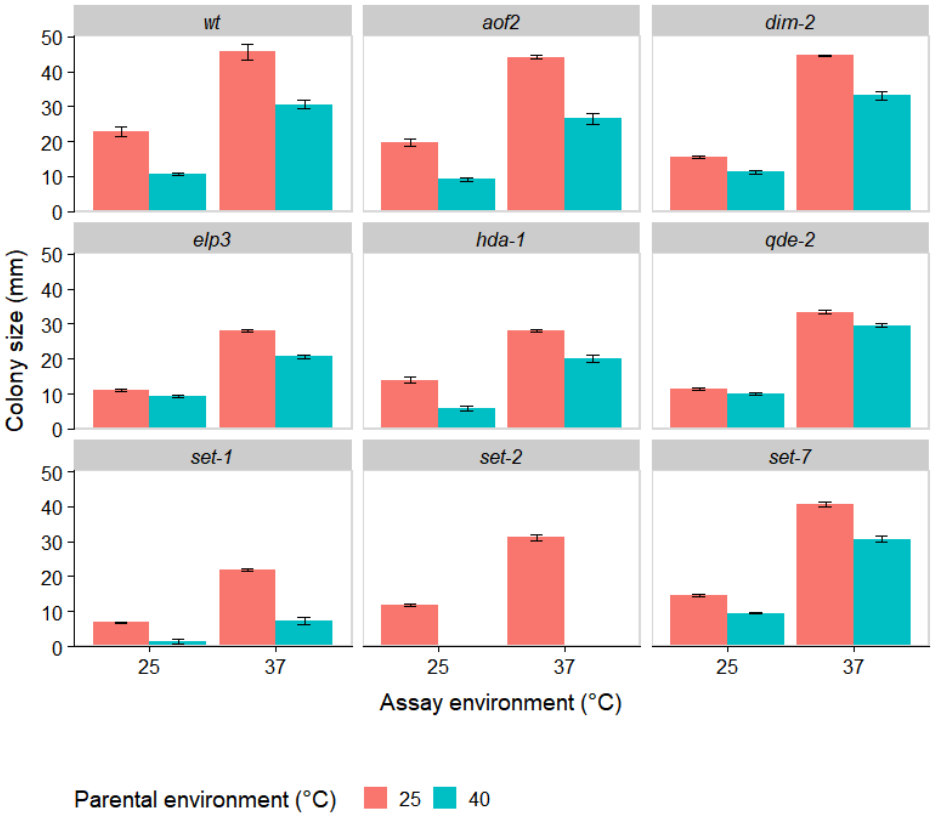

Figure 4. Colony size for strains in temperature experiment 2. Error bars represent standard error or the mean.

Table 7. Statistical results of the effect of parental environment on colony size temperature experiment 3. Difference shows the effect of parental environment on colony size, when the former parental environment is compared to the latter.

Strain	Compared parental environments	Assay environment	Difference	Tukey p
wt	40 °C and 25 °C	25 °C	-0.14	1.00
wt	40 °C and 25 °C	37 °C	1.36	0.997
dcl-1 dcl-2	40 °C and 25 °C	25 °C	-1.93	0.952
dcl-1 dcl-2	40 °C and 25 °C	37 °C	-1.29	0.998
nst-1	40 °C and 25 °C	25 °C	-6.71	< 0.001
nst-1	40 °C and 25 °C	37 °C	-5.07	0.016

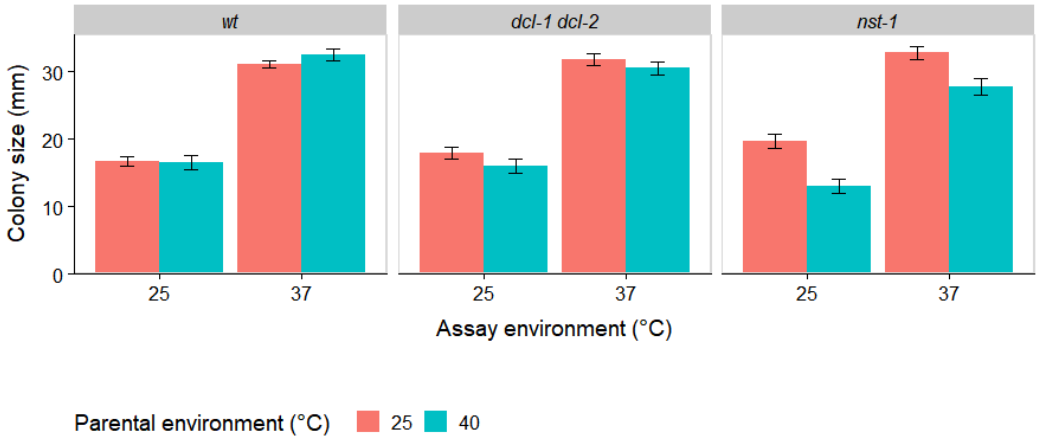

Figure 5. Colony size for strains in temperature experiment 3. Error bars represent standard error of the mean.

3.2.2 Spore size

In sucrose experiment 1, spores produced in the parental environment 0.015% were larger (Table 8 and Figure 6). In the temperature experiments 1 + 3 (combined data), spores produced in parental environment 40 °C were larger for strains *wt* and *nst-1* (Table 9 and Figure 7). For *dcl-1 dcl-2,* there was no size difference between spores produced in different parental environments. ANOVA table is supplied in Appendix 1, Table 13.

If the data from temperature experiments 1 and 3 were analysed separately, spore size in temperature experiment 1 was statistically significantly larger in parental environment 40 °C. In the temperature experiment 3, the spore size in different parental environments was statistically significantly different for strains *nst-1* and *dcl-1 dcl-2*. For these strains, larger spores were produced in parental environment 40 °C. Data from this alternative analysis is not shown..

Table 8. Statistical results of the effect of parental environment on spore size in the sucrose experiment 1. PE = parental environment.

Coefficients	**Estimate**	**Standard error**	**t-value**	**p-value**
Intercept	5.30	0.04	125.61	< 0.001
PE 0.015%	0.18	0.06	2.97	0.006

Table 9. Statistical results of the effect of parental environment on spore size for each strain in temperature experiments 1 + 3 (combined data). Difference shows the effect of parental environment on spore size, when the former parental environment is compared to the latter.

Strain	Compared parental environments	Difference	Tukey p
wt	40 °C and 25 °C	0.27	< 0.001
dcl-1 dcl-2	40 °C and 25 °C	0.21	0.199
nst-1	40 °C and 25 °C	0.49	< 0.001

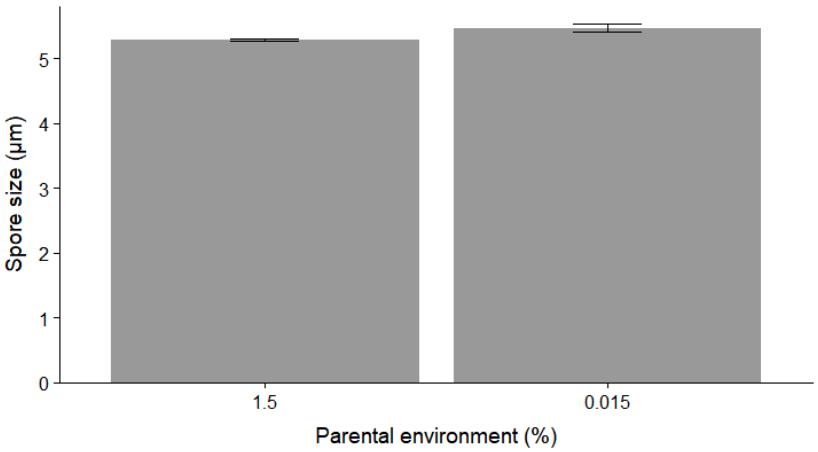

Figure 6. Spore size in different parental environments in sucrose experiment 1. Error bars represent standard error of the mean.

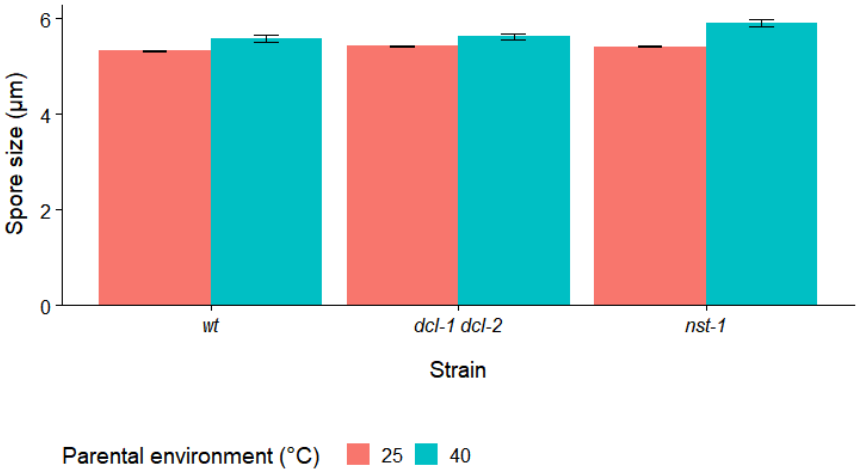

Figure 7. Spore size in different parental environments in the temperature experiments 1 + 3 (combined data). Error bars represent standard error of the mean.

3.3 Competition experiment

The offspring whose parents were from 1.5% sucrose performed better than those whose parents were from 0.015% (relative fitness of offspring with parental environment 1.5% = 9.80, 7.93–12.6 95% HPDI, Figure 8B). Mating type *A* had a lower fitness in comparison to mating type *a* (relative fitness of *mat A* = 0.626, 0.553–0.705 95% HPDI, Figure 8A). The csr-tag itself also had a small adverse effect on relative fitness (relative fitness of csr*-tagged strain = 0.881, 0.782–0.992 95% HPDI, Figure 8A). Figure 9 shows the proportions of marked strain at the end of competition in different competition environments and for different competitors.

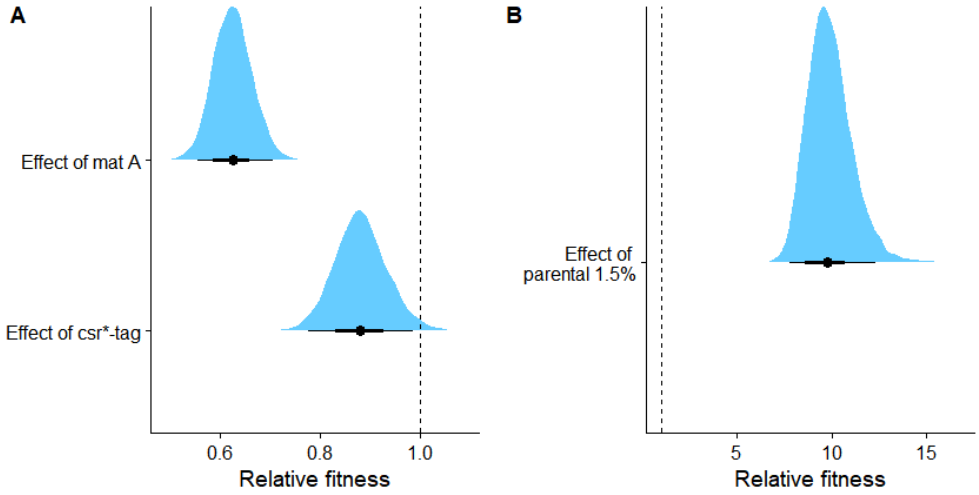

Figure 8. A) Effects of *mat A* and csr*-tag on relative fitness. B) Effect of parental environment 1.5% on relative fitness. The dashed line marks the point where the competitive fitnesses of the competitors are equal. Blue areas show the posterior distributions, while thin and thick black lines show the 95% and 66% HPDI, respectively.

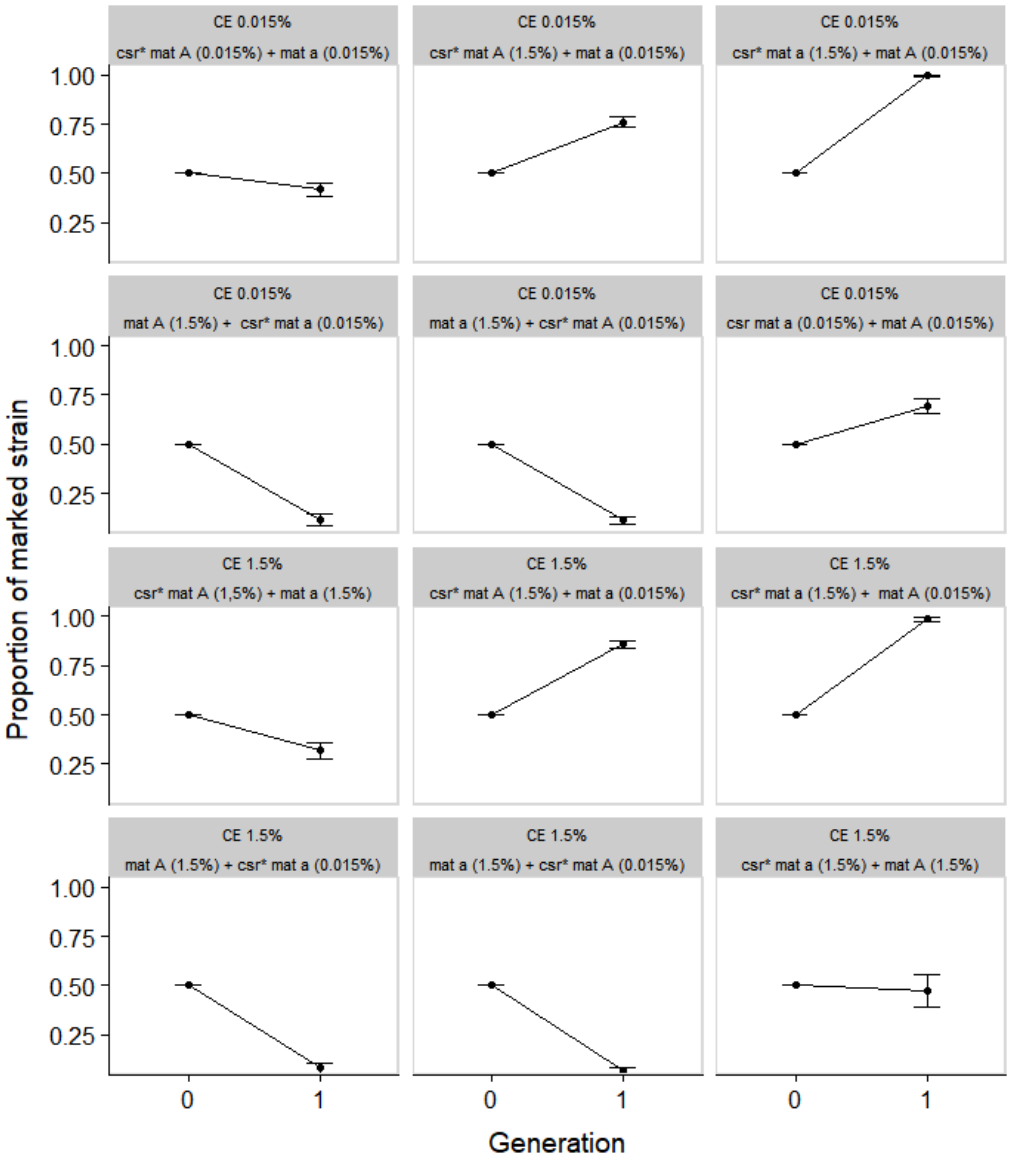

Figure 9. The proportion of marked strain after competition. CE = competition environment. Generation = number of generations the strains were competing. The numbers inside parentheses are the parental environments for each competitor. Error bars represent standard error of the mean.

4 DISCUSSION

4.1 Existence of between-generation effects

Between-generation effects in relation to both sucrose concentration and temperature seem to exist in *N. crassa*. If the parental generation came from

environments 0.015% sucrose or 40 °C, offspring growth was reduced in both assay environments in comparison to parental environments 1.5% sucrose and 25 °C. The direct environment also had an effect on offspring growth. Offspring grew better in assay environments 1.5% and 37 °C than in 0.015% or 25 °C, respectively. Based on these results, the properties of the parental environment seems to be more important to offspring performance in *N. crassa* than the match between the parental and offspring environments, which is often discussed in relation to adaptive between-generation effects (Uller et al. 2013, Engqvist and Reinhold 2016). In my experiment, the offspring didn't get any benefits for a life in 0.015% sucrose if the parents also came from these conditions. The same seemed to be true for temperature, even if a match between parental and assay environments were not achieved here. Therefore the between-generation effects found here do not seem adaptive in the same sense as described e.g. by Galloway and Etterson (2007). Instead, my results indicate the presence of a silver-spoon effect generated by parental environments 1.5% and 25 °C.

4.2 Transmission of between-generation effects

4.2.1 Spore size

In relation to both sucrose concentration and temperature, spores produced in parental environments 0.015% and 40 °C were larger, except for strain *dcl-1 dcl-2*. For this strain, there was no difference in spore size between the parental environments. The results from the alternative analyses were different only in terms of statistical significance of the spore size difference between parental environments in strain *dcl-1 dcl-1*, and so not further discussed here. As shown in the previous sections, environments 0.015% and 40 °C didn't seem to produce any between-generation effects. Therefore, this result for spore size difference is somewhat in conflict with other results obtained in this study. There could be many causes for such discrepancy, but three possibilities are: (1) provisioning via spore size is not the transmission mechanism behind between-generation effects, (2) the effects of provisioning were overruled by a stronger mechanism acting through

environments 1.5% and 25 °C, or (3) spores produced by parents in environments 1.5% and 25 °C had higher energy/nutrient density. Based on this study, none of these possibilities can be considered more likely than the other. However, studies where parents produced larger offspring in environments with less food resources do exist (Garbutt and Little 2017), and so the results gained here in terms of spore size are plausible. Thus, provisioning can't be excluded as a possible source of the between-generation effects.

4.2.2 Epigenetic inheritance

Mutant strains showed rather similar growth patterns than the wild-type in relation to both sucrose and temperature. The only strain that showed a different growth pattern was *set-2,* which grew better in G3 if parents came from parental environment 0.015%. The differences between parental environments were also not statistically significant for this strain, and so this result could be an indication that the histone modification mutation of *set-2* (H3K36me) might make it unable to produce the between-generation effect. Loss of function in *set-2* in *N. crassa* causes increased transcription in some genes and decreased transcription in others (Bicocca et al. 2018), and among other deficiencies strains lacking *set-2* function are also unable to reproduce sexually as a female (Adhvaryu et al. 2005). Reproductive deficiencies have also been observed in *C. elegans* in response to H3K36me3 deficiency (Kreher et al. 2018). If the loss of H3K36me3 was caused by mutation in gene *met-1* in *C. elegans*, the proportion of sterile worms was dependent on temperature. In my study, strain *set-2* didn't grow at all if the parents were exposed to 40 °C. This indicates that sterility could be influenced by temperature also in *N. crassa,* and therefore that methylation of histone H3K36 is especially important in high temperature. As all *N. crassa* samples were reproducing asexually in my experiments, this sterility in high temperature seems not to be explained by the female sterility observed by Adhvaryu et al. (2005). *Set-2* growth deficiency in high temperatures has also been observed by Kronholm et al. (2016).

Other mutant strains that didn't show statistically significant difference between parental environments in either assay environment were *hda-1* in relation to sucrose and *qde-2* and *dcl-1 dcl-2* in relation to temperature. One way or the other, the mutations in these strains seem to be involved in gene silencing (Catalanotto et al. 2004, Smith et al. 2010). Based on this, it is implicated that silencing of some genes is required for between-generation effects to emerge. The results indicating the involvement of *dcl-1 dcl-2* in the production of the between-generation effect should be treated with caution, however, as this strain was part of the temperature experiment 3, where also the wild-type control seemed to have no differences between the parental environments (see section 3.2.1).

The reason why some strains (*dcl-1 dcl-2* in the sucrose experiment 2 and *elp3* and *dim-2* in the temperature experiment 2) showed a statistically non-significant difference between the parental environments only in one assay environment might be the assay environment itself. All of the non-significant differences were measured in the assay environment where growth was slower, and therefore the slower growth itself might have contributed to smaller size differences between colonies. However, I see no reason to assume that a certain mutation necessarily produces similar effects in all levels of an environmental factor. Therefore, it could be possible that certain epigenetic modification is involved in producing the between-generation effect e.g. in 1.5% sucrose, but not in 0.015%.

4.3 Competition experiment

In laboratory environment, between-generation effects are sufficient to provide the offspring significant competitive benefits. Though mating type and the csr*-tag itself had some fitness effects as well, the benefit provided by the parental environment was many times larger. This should be sufficient to provide offspring benefits even in the wild and in the presence of other selective pressures, and thus indicates that the ability to produce between-generation effects might be under positive selection. *N. crassa* has been shown to be a rather weak competitor, as its ability to colonize unsterilized surfaces is poor (Lee 2012). In this context the

competitive benefit provided by the between-generation effects could be even more important. They could allow *N. crassa* to grow fast into a large colony on favourable surface and thus leave no room for competitors. As *N. crassa* seems to have no trade-off between growth and asexual spore production (Anderson et al. 2019), fast growth and large reproductive effort could be possible simultaneously.

4.4 Limitations of this study

Because this study didn't extend beyond one generation since the parental (exposed) generation, it cannot exclude the possibility that the observed growth differences in relation to parental generation were the result of direct exposure of the offspring to these conditions as spores. It has been pointed out in literature, that to conclusively show the existence of a true transgenerational effect, these effects would need to be demonstrated with a generation that was not exposed to the parental conditions at any time during its lifespan (Skinner 2008). There also seem to be variation in the results between different sub experiments. In the temperature experiment 3 there seems to be next to no difference between the parental environmental effects for the wild-type and *dcl-1 dcl-2*. Because this experiment were conducted identically to the other mutant experiments, excluding the number of biological replicates, in the bounds of this study there is no explanation for these results. The sheer number of biological replicates is unlikely to be the cause. The lack of filtration described in section 2.3.1 for sucrose experiment 1, temperature experiment 1 and competition experiment didn't seem to have an effect on the results.

4.5 Conclusions

In this study, I have found that between-generation effects exist in *N. crassa* in a form of silver-spoon effects, in response to both sucrose concentration in the growth medium and temperature. In direct competition, offspring with parents from 1.5% sucrose gained a substantial increase in competitive fitness over those whose parents came from 0.015% sucrose. Multiple epigenetic modifications were

indicated as a possible source, but provisioning was not excluded. These results show that between-generation effects can be important among fungi and a source of phenotypic variation.

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APPENDIX 1. ANOVA tables

Table 10. ANOVA table of the model for colony size in sucrose experiment 2 for. PE = parental environment. AE = assay environment.

	Df	Sum of squares	Mean sum of squares	F	p-value
PE	1	906.86	906.86	801.43	< 0.001
AE	1	114.33	114.33	101.04	< 0.001
Strain	10	888.81	88.88	78.55	< 0.001
PE × AE	1	23.57	23.57	20.83	< 0.001
PE × strain	10	244.05	24.40	21.57	< 0.001
AE × strain	10	51.11	5.11	4.52	< 0.001
PE × AE × strain	10	19.19	1.92	1.70	0.081
Residuals	292	330.41	1.13		

Table 11. ANOVA table of the model for colony size in the temperature experiment 2. PE = parental environment. AE = assay environment.

	Df	Sum of squares	Mean sum of squares	F	p-value
PE	1	3388.68	3388.67	823.28	< 0.001
AE	1	20316.05	20316.05	4935.79	< 0.001
Strain	8	6971.57	871.45	211.72	< 0.001
PE × AE	1	295.25	295.25	71.73	< 0.001
PE × strain	7	744.78	106.40	25.85	< 0.001
AE × strain	8	1391.62	173.95	42.26	< 0.001
PE × AE × strain	7	110.62	15.80	3.84	< 0.001
Residuals	201	827.33	4.12		

Table 12. ANOVA table of the model for colony size in the temperature experiment 3. PE = parental environment. AE = assay environment.

	Df	**Sum of squares**	**Mean sum of squares**	**F**	**p-value**
PE	1	110.86	110.86	17.59	< 0.001
AE	1	4364.65	4364.65	692.63	< 0.001
Strain	2	12.21	6.11	0.97	0.3843
PE × AE	1	8.36	8.36	1.33	0.2532
PE × strain	2	152.88	76.44	12.13	< 0.001
AE × strain	2	6.38	3.19	0.51	0.605
PE × AE × strain	2	1.02	0.51	0.08	0.922
Residuals	72	453.71	6.30		

Table 13. ANOVA table of the model for spore size in temperature experiments 1 + 3 (combined data). PE = parental environment. AE = assay environment.

	Df	**Sum of squares**	**Mean sum of squares**	**F**	**p-value**
Strain	2	0.82	0.41	7.24	0.001
PE	1	2.62	2.62	46.60	< 0.001
Strain × PE	2	0.32	0.16	2.86	0.062
Residuals	106	5.97	0.06		

APPENDIX 2. The standard curve

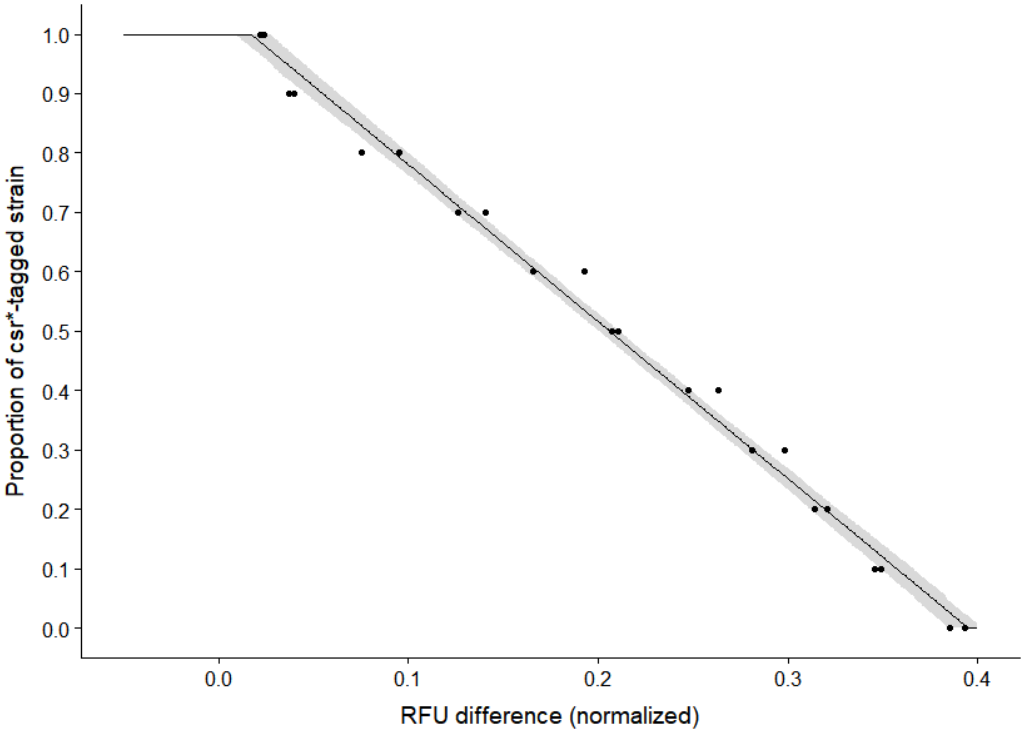

Figure 10. The standard curve used for calculating the amount of csr*-tagged strain in the sample.