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Parental effects in a filamentous fungus: Phenotype, fitness and mechanism

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Abstract

In nature, organisms have to cope with constantly changing environments. In certain conditions, it may be advantageous for the parents to pass on information about the environment, or resources to their offspring. Such transfers are known as parental effects, and they are well documented in plants and animals, but not in other eukaryotes, such as fungi. Many fungi disperse through spores, and fungal spores can potentially carry information or resources to the next generation. Understanding parental effects and their evolutionary consequences in fungi is of vital importance as they perform crucial ecosystem functions. In this study, we investigated whether parental effects are present in the filamentous fungus *Neurospora crassa*, how long do they last, whether the effects are adaptive, and what is their mechanism. We performed a fully factorial match/mismatch experiment for a good and a poor quality environment, in which we measured the initial growth of strains that experienced either a matched or mismatched environment in their previous generation. We found a strong silver-spoon effect in initial mycelium growth, which lasted for one generation, and increased fitness during competition experiments. By using deletion mutants that lacked key genes in epigenetic processes, we show that epigenetic mechanisms are not involved in this effect. Instead, we show that spore glycogen content, glucose availability and a radical transcription shift in spores are the main mechanisms behind this parental effect.

KEYWORDS
fungal spore, fungi, intergenerational effect, *Neurospora crassa*, transgenerational effect

1 | INTRODUCTION

Organisms must adjust their phenotype throughout development to match the current environment. Phenotypic adjustments based on parental cues or resources may be especially important during early stages of life, when the organism cannot yet forage or obtain cues from the environment as efficiently as the parent. The effect that the parental phenotype or environment has on the fitness of the offspring, that is not explained by direct genetic transmission, is known as a parental effect (Badyaev & Uller, 2009; Reddon, 2012).

Parental effects are usually studied using match/mismatch experiments. These are fully factorial reciprocal transplant experiments

where the offspring performance is measured in the same or different conditions compared to their parental environment (Engqvist & Reinhold, 2016). This experimental design is necessary to determine the existence and the type of parental effects. One type of parental effect is the anticipatory effect, where offspring performance is greater when their own environment matches the environment of their parent, and the offspring adjust their phenotype in an adaptive manner. Another possible outcome is a silver-spoon effect. This happens when the quality of the parents, or the parental environment, is the main factor that shapes offspring fitness. Parental effects can also be a combination of anticipatory effects and silver-spoon effects (Engqvist & Reinhold, 2016).

There are many examples of parental effects in plants and animals. For example, in the crustacean *Daphnia* a signal perceived by the mother that induces the development of a defensive structure can be inherited from mothers to offspring (Agrawal et al., 1999). In the small brown planthopper, the parental generation that was exposed to predator odour cues gave rise to a higher proportion long-winged females, which present lower predation rate, in comparison to non-exposed parents (Wen & Ueno, 2021). Also in the solitary bee, *Osmia lignaria* the exposure to pesticide reduces reproductive success in the next generation (Stuligross & Williams, 2021). In the plant *Arabidopsis*, stress experienced by the parents can prime their offspring to better cope with osmotic stress (Wibowo et al., 2016), or pathogens (Slaughter et al., 2012). Parental effects have also been shown to contribute to adaptation (Dey et al., 2016; Kilner et al., 2015; Lind et al., 2020).

Parental effects can be transmitted via several mechanisms. One of them is the quality and quantity of provisional molecules such as nutrient reserves, mRNAs and proteins. These supplies could be altered by the parental condition and significantly impact the offspring's performance either at early, or all stages along its lifetime (Dyer et al., 2010; Herman & Sultan, 2011; Moles & Westoby, 2006). Also, parental conditions can induce epigenetic changes (e.g. DNA methylation and histone modifications), which can be inherited in some cases and influence gene expression and phenotypic traits (Bošković & Rando, 2018; Herman & Sultan, 2011, 2016; Jablonka & Raz, 2009; Wibowo et al., 2016). For instance, in dandelions DNA methylation patterns induced by environmental stressors can be transmitted to the next generation even when the stressor is removed from the offspring environment (Verhoeven et al., 2010). In plants, the induced responses are thought to be transmitted via the RNA directed DNA methylation pathway, as it has been implicated in inherited parental effects (Luna & Ton, 2012; Wibowo et al., 2016). The mechanism behind the parental effects, regardless of the type (i.e. anticipatory effects or silver-spoon effets), may influence their duration. If the underlying mechanism is epigenetic, the parental effect may persist across generations, while a provisioning effect may be only brief (Herman & Sultan, 2011). Even though parental effects have been widely studied, the underlying mechanisms are rarely documented (Sánchez-Tójar et al., 2020). To understand how parental effects aid adaptation it is crucial to first understand under which circumstances parental effects manifest, are they transmitted and what are their underlying mechanisms.

So far, most of the research on parental effects has focused on plants and animals, and to date just a few a studies have investigated the existence of parental effects in microbes. Even though theoretical models suggest that the ability to respond to the environment via an anticipatory effect is expected to evolve when environmental fluctuations span several generations, which may be often true for microbes (Kronholm, 2022). To our knowledge only one previous study has investigated maternal effects in a fungus. Zimmerman et al. (2016) reported the existence of asymmetrical investment in the fungus *Neurospora crassa*. The authors discovered that, when the fungus reproduces sexually, maternal effects influences spore number and germination success (Zimmerman et al., 2016). The lack of research of parental effects on fungi is surprising, as they perform key ecosystem functions such as organic matter decomposition and are involved in plant symbiosis (Bahram & Netherway, 2022).

To understand parental effects in fungi, we investigated the existence and mechanisms of parental effects in the filamentous fungus *Neurospora crassa*. *N. crassa* has a facultative sexual cycle, but we focused on parental effects that are transmitted through asexual spores, called conidia. Fungal spores can potentially contain molecules deposited by the parent that influence the performance of the offspring. We also determined the fitness relevance and duration of such effects. Finally, we investigated the mechanisms behind the parental effects by quantifying nutrient reserves, using mutants and RNA-seq. Our study is one of the first to thoroughly examine parental effects in fungi.

2 | METHODS

2.1 | Existence of parental effects

To investigate whether parental effects exist in *Neurospora crassa* we performed a reciprocal match/mismatch experiment (Engqvist & Reinhold, 2016). We compared the initial colony size after inoculation in two different environments where the strains had experienced either the same or a different environment in the previous generation (Figure 1a). We inoculated conidia of *N. crassa* strain 2489, obtained from Fungal Genetics Stock Center (McCluskey et al., 2010), in slants containing Vogel's medium N (VM) (Metzenberg, 2003) with either 1.5% or 0.015% sucrose. The fungus grew in the slants for one generation, defined here as growing from spore to spore. Each slant represented a biological replicate. At the end of generation one, conidia were harvested and filtered to remove any mycelial fragments, then counted and measured using a CASY cell counter with a 45 μm capillary and a gating window of 2.5–10 μm. We inoculated 10,000 conidia at the center of a petri dish with VM agar, containing the parental or a different sucrose concentration. We inoculated 3–4 plates from each slant. Plates were randomized and incubated at 25°C. We

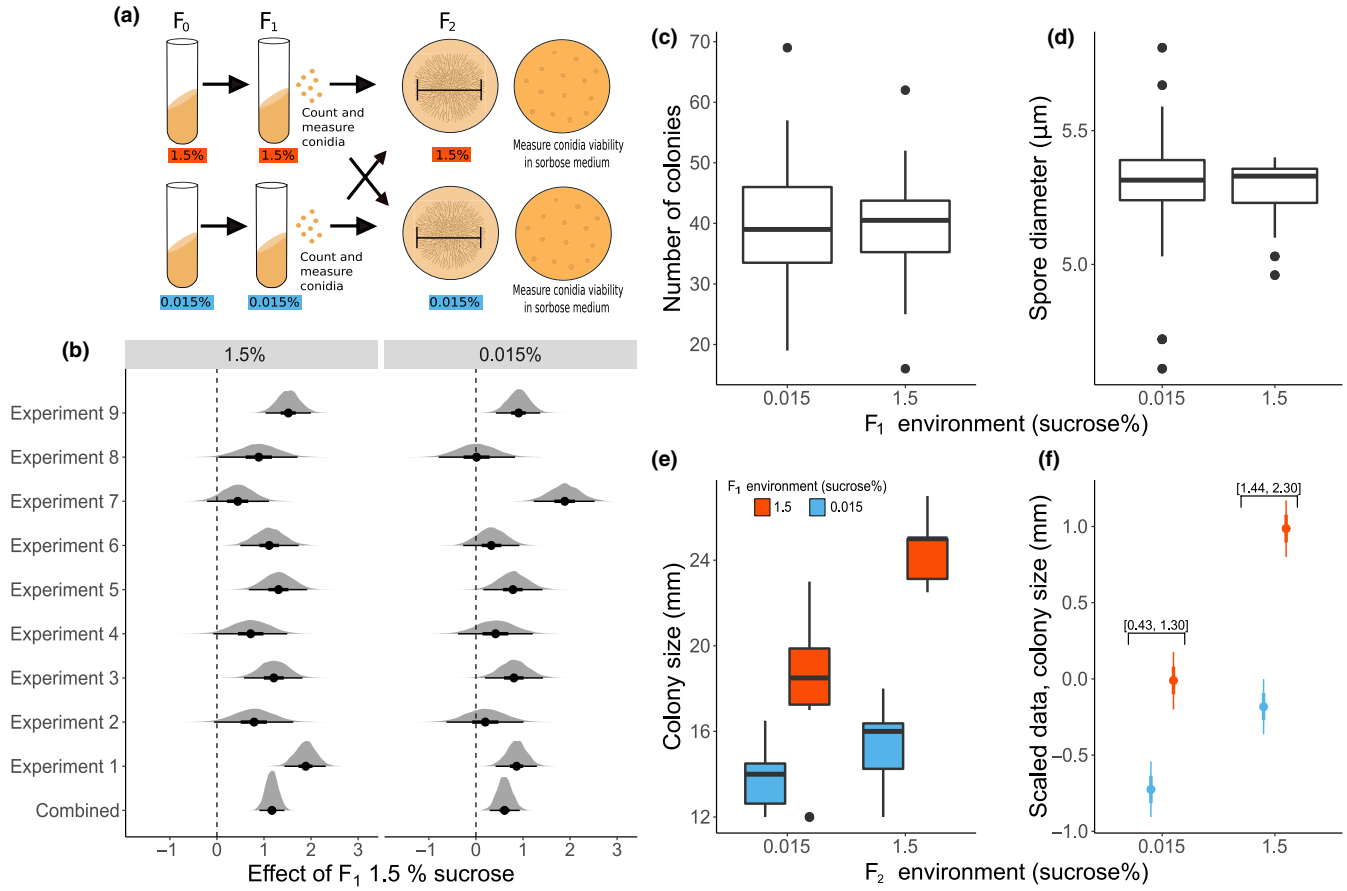

FIGURE 1 Effect of F_1 sucrose concentration on colony size in F_2. (a) Experimental design. Fungus was cultivated in 1.5% or 0.015% sucrose slants for two generations, then the same number of conidia were plated on plates with 1.5% or 0.015% sucrose. The colony size was measured and the number of colonies formed in sorbose plates was counted to estimate spore viability. (b) Posterior distributions of the effect of F_1 1.5% sucrose on initial colony size, when F_2 was grown in either 1.5% or 0.015% sucrose. Sample size of each experiment is reported in Table S1. (c) Number of colonies in sorbose plates. (d) Conidial diameter. (e) Raw data of initial colony size from experiment one. (f) Model estimates using the combined data. The 95% HPDI of the difference between treatments is shown in square brackets.

measured the diameter of the mycelial colony at three time points: first after 14–18 h from inoculation, then second and third measurements 2–4 h after the previous measurement. Growth rate was estimated as the slope of the linear regression of time against colony diameter. To make sure that differences in colony size was not driven by spore viability or dormancy, we measured conidial viability by plating the harvested conidia on sorbose medium. Sorbose induces colonial morphology in *N. crassa* (Davis & de Serres, 1970), this allowed us to count the number of germinated conidia after 3 days of incubation at room temperature. The experiment was repeated nine times, sample sizes for each experiment are reported in Table S1.

We also explored the existence of parental effects on alternative carbon sources. We performed a match/mismatch experiment where we compared sucrose to an alternative carbon source: cellulose, lactose, maltose or xylose. We measured initial colony size when the fungus was exposed to either the same or a different carbon source in the previous generation. There were five biological replicates for each treatment.

To investigate if the parental environment has an impact on the germination rate, we performed the same match/mismatch experiment described above (Figure 1a), with the difference that 100,000 conidia were inoculated and left to germinate in the wells of a chambered cover slide (ibidi cat no 81507) containing 40 uL of liquid VM with either 1.5% or 0.015% sucrose. We had six slants (biological replicates) per treatment. With the inverted the Leica Dmi8 inverted microscope we photographed three randomized locations each hour for 6 h. In each photo we counted the total number of conidia and the number of germinated conidia. We considered a conidium germinated when the germ tube was clearly visible. We only counted spores that were complete and on focus.

2.2 | Transmission of parental effects

To investigate whether the parental effects persisted for more than one generation we continued the experiment described above, into the third generation. At the end of the second generation, conidia were harvested, counted and plated. Mycelial growth was measured in plates that either matched or mismatched the F_1 sucrose environment (Figure 2a). We assessed conidia viability as above. We

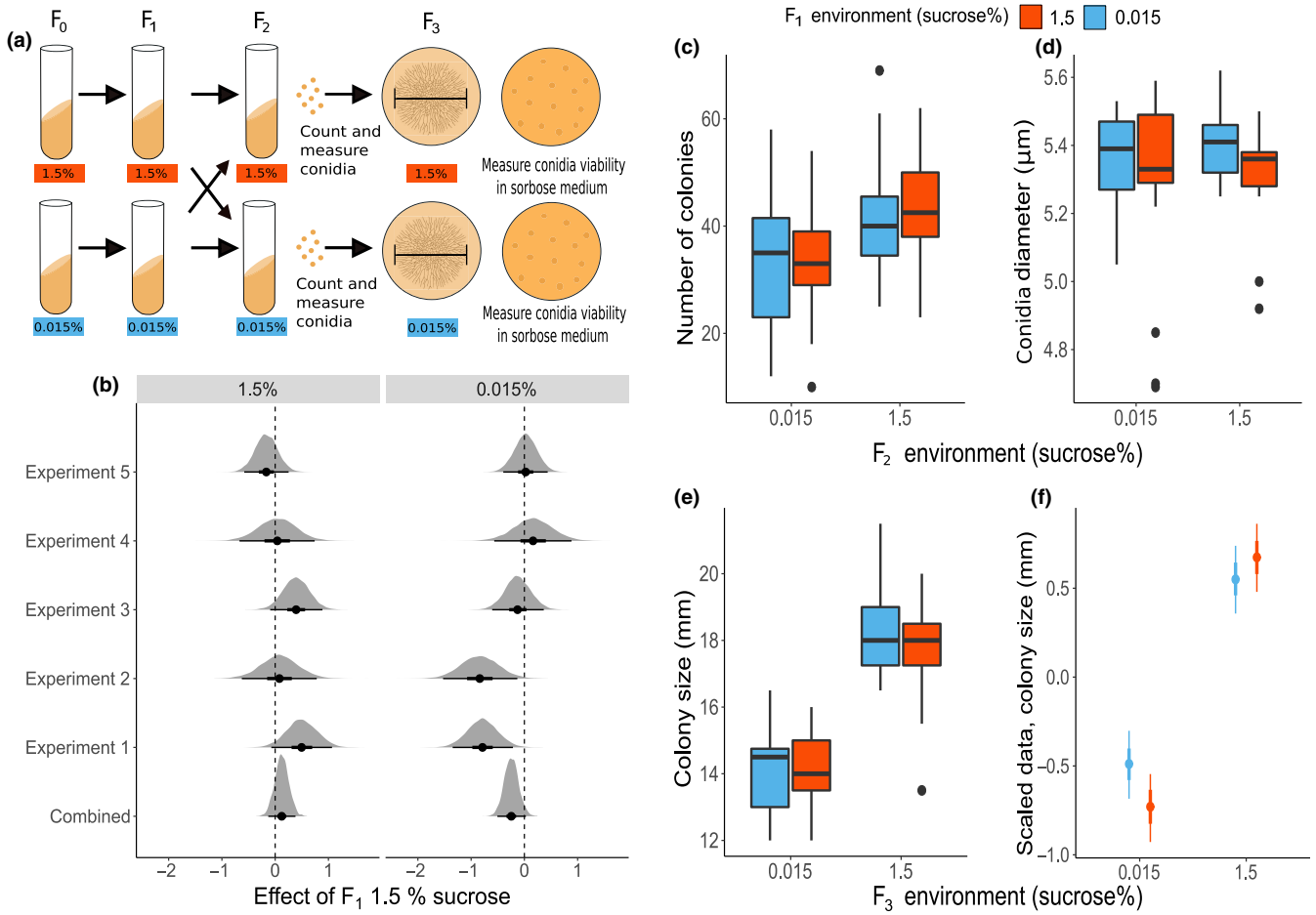

FIGURE 2 Effect of F_1 sucrose concentration on colony size in F_3. (a) Experimental design. Fungus was cultivated in 1.5% or 0.015% sucrose slants for two generations, then matched or mismatched for one generation, and then the same number of conidia were plated on plates with 1.5% or 0.015% sucrose. The colony size was measured and the number of colonies formed in sorbose plates was counted to estimate spore viability. (b) Posterior distributions of the effect of F_1 1.5% sucrose on initial colony size, when F_3 was grown in either 1.5% or 0.015% sucrose. Sample size of each experiment are reported in Table S1. (c) Number of colonies in sorbose plates. (d) Conidial diameter. (e) Raw data of initial colony size from experiment one. (f) Model estimates using the combined data.

repeated this experiment five times, the sample size of each experiment is reported in Table S1.

2.3 | Fitness consequences of parental effects

To estimate the fitness effects that the parental environments cause, we used a competition experiment with marked strains. We have previously developed marked strains for *N. crassa* by inserting a DNA barcode to the *csr*-locus, this marker allows us to estimate the proportion of the marked strain in a sample of conidia using high resolution melting (HRM) PCR (Kronholm et al., 2020). The experimental design was the same as in the initial match/mismatch experiment, except instead of plating conidia at the F_2 generation, we combined conidia from two different strains in a slant, and let them produce conidia in competition. Then, we estimated the proportion of the marked strain in the conidial sample using HRM-PCR (Figure S1A). We have previously observed that the mating-type locus and the *csr*-tag have fitness effects, so in order to estimate the fitness effect of the parental sucrose environment we combined the parental environment, competition environment, the *csr*-tag and the mating-type locus in 8 different combinations (Table S2). Strains with the same mating-type were never competed against each other, because in these nearly isogenic strains, hyphae of the same mating-type would fuse together and no competition would occur (Kronholm et al., 2020). A detailed description of the competition experiments can be found in Methods S1.

2.4 | Mechanisms of parental effects

2.4.1 | Protein content and carbohydrate reserves

To investigate qualitative differences in the conidia, we assayed whether protein, glycogen, or glucose reserves differed between F_2 conidia coming from 0.015% or 1.5% sucrose. We measured protein and sugars using kits: BCA protein assay kit (Thermo Scientific), glycogen assay kit (Sigma-Aldrich, MAK016) and glucose assay kit

(Sigma-Aldrich, MAK263), according to manufacturers' instructions. We extracted total protein from 40 million conida, and glycogen and glucose from 70 million conidia (see Methods S1).

2.4.2 | RNA-seq of conidia

To understand the mechanisms behind parental effects we investigated the gene expression patterns of conidia coming from either 1.5 or 0.015% sucrose. We extracted RNA from conidia following (Kramer, 2007). See Methods S1 for details and Table S3 for purity, concentration and integrity metrics of the extracted RNA. We used the ERCC RNA Spike-ins (Lemire et al., 2011) as external controls (see Methods S1). Six biological replicates from each sucrose concentration were sent to Novogene (Cambridge, UK) for mRNA poly A enrichment library preparation and transcriptome sequencing, using the Illumina NovaSeq platform with 150 bp paired-end libraries.

2.4.3 | RNA-seq normalization and analysis

We examined the quality control metrics of the 12 sequenced samples with FastQC. The samples were aligned against *N. crassa* reference genome (assembly NC12) with the added 92 ERCC RNA Spike-In control transcripts. We aligned the sequences using hisat (Kim et al., 2019) specifying 2500 as the maximum intron length (Cemel et al., 2017), and all other parameters were set as default. The number of obtained reads and alignment metrics are reported in the Table S3.

We normalized the data using the trimmed mean of the M-values approach, and then used the ERCC spike-in controls to remove unwanted variation using the RUVseq package (Risso et al., 2014). Finally, we used DESEq2 to obtain differentially expressed genes and cluster profiles to perform an over-representation analysis (ORA) and a gene set enrichment analysis (GSEA). See Methods S1 for details.

2.4.4 | Epigenetic mechanisms

To investigate whether the observed silver-spoon effect relied on epigenetic mechanisms, we performed the same basic match/mismatch experimental design (Figure 1a), but with three deletion mutants deficient for different epigenetic mechanisms. The mutants were as follows: Δ*dim-2*, which lacks DNA methylation (Kouzminova & Selker, 2001); Δ*qde-2*, which has compromised RNA interference pathway (Dang et al., 2011); and Δ*set-7*, which lacks trimethylation of the lysine 27 on the histone 3 (H3K27me3) (Jamieson et al., 2013). The sample size was $n = 40$ plates for each mutant strain. The mutant strains have been previously described in Kronholm et al. (2016).

We further explored the overlap between the genes belonging to the main GSEA-enriched pathways and different genomic domains. In *N. crassa*, the trimethylation of histone 3 lysine 9 (H3K9me3) is associated with heterochromatin, H3K27me3 with facultative heterochromatin and the dimethylation of histone 3 lysine 36 (H3K36me2) with euchromatin. DNA methylation occurs only in H3K9me3 domains. Briefly, we obtained ChIP-seq reads for each of the domains, we aligned them to the reference genome and identified the domains of the histone modifications (see Methods S1). Then, we identified the intersecting regions between each histone modification domains and the genes from each GSEA-enriched pathway. We considered a gene to belong to a histone modification domain if at least 20% of the gene overlapped with the histone modification domain.

2.5 | Statistical analyses

2.5.1 | Existence and duration of parental effects

Since time of the first measurement varied between experiments, the data were centered and variance standardized experiment by experiment by subtracting the mean and dividing each observation by the standard deviation. We fitted Bayesian models using Hamiltonian Monte Carlo implemented with the Stan language (Carpenter et al., 2017) using the "ulam" function available in the rethinking package (McElreath, 2020), in R version 4.0.2. We fitted a model with initial colony size as response, treatment and spore viability as predictors, and the slant as a random factor. See Methods S1 for details. The estimates and the highest posterior density intervals (HPDI) of all models are reported in the Table S4.

For both F_2 and F_3 data, we analysed each experiment separately and for all experiments combined (Figures 1b and 2b). When analysing each experiment independently, we did not considered slant (β_s) an viability (β_c) in the model because the sample was not big enough for the model to converge. When analysing initial colony size of F_2, we did not considered viability (β_c) in the model as it did not have a significant effect, and three experiments had missing viability data.

The effect of the parental environment on germination rate was modelled by fitting a binomial regression (Figure S2A) using the "brm" function available in the brms package (Bürkner, 2018). We considered the number of germinated spores in each sample as a response variable, condition, and the interaction between time and condition as predictors and time and slant as group-level terms. See model details in the Equation S3. We only considered data up to hour four mycelium growth in further hours impedes counting the spores with confidence. The estimates and the HPDI for this model is reported in the Table S5.

2.5.2 | Fitness consequences of parental effects

We estimated the relative fitness effect of the parental 1.5% sucrose environment following the same principle as in Kronholm et al. (2020). We used a model that takes the uncertainty in proportion estimates of the marked strain into account and models the log-ratio of the

strain proportions. With this model specification the slope of the model is the log of relative fitness (Kronholm et al., 2020). The log-ratio of the strain proportions was the response, the effect of *csr-1*⁺* marker, mating-type and parental environment were predictors, and population was a random factor. See Methods S1 for details.

3 | RESULTS

3.1 | Existence of parental effects

To test if different parental resources cause parental effects in *N. crassa*, we performed a reciprocal match/mismatch experiment with a rich (1.5%) and a poor (0.015%) sucrose environment. All the results from the Bayesian model in Equation S1 are reported as means with 95% highest posterior density intervals (HPDI) in square brackets. We observed that the initial colony size was always higher in the 1.5% sucrose environment (Figure 1e,f). Also, if the fungus experienced 1.5% sucrose in the previous generation, the initial size was higher regardless of the F_2 assay environment. The initial colony size of the fungus coming from the high sucrose environment was 17% bigger when growing in 1.5% and 10% bigger when growing in 0.015% sucrose, both compared to the fungus growing in the same sucrose concentration but which experienced 0.015% sucrose in the previous generation. The difference for F_1 treatments was 1.167 [0.913, 1.425] when grown in 1.5% sucrose, and 0.714 [0.450, 0.970] when grown in 0.015% sucrose. Since the parental environment with 1.5% sucrose always produces larger colonies in the next generation no matter what the current environment is, the parental effects observed here are due to 1.5% sucrose just being a better environment overall, with no evidence of any adaptive response to low resources by the fungus. This type of parental effect is also called a silver-spoon effect, since an individual growing in a better environment will always be better off (Bonduriansky & Crean, 2018).

We repeated the experiment nine times. We observed some variation in experimental outcomes for unknown reasons. In some of the experiments, the effect of the F_1 environment overlapped with zero but when data from all experiments were combined and analysed together parental environment clearly affected colony size in the next generation (Figure 1b,f).

To verify that the silver-spoon effect relies on differences in growth and not on differences in germination, we measured the germination rate on spores inoculated in an environment that matched or mismatched the parental conditions. We found that the spores germinating and coming from 0.015% sucrose environment germinated the fastest being the median of the time to reach 50% germination (i.e. median germination) 2.84 h (Figure S2B). Spores germinating and coming from 1.5% environment were the second fastest to germinate with a median germination of 3.13 h. The difference between these two treatments was −1.919 [−2.696, −1.088]. The spores coming from a 1.5% environment germinating in 0.015% sucrose environment were the slowest to germinate being the median germination of 3.6 h. When the spores were germinating in 1.5% sucrose, the parental environment did not affect germination, the difference between treatments is −0.156 [−0.931, 0.568]. However, when the spores were growing at 0.015% the parental environment affected germination, the difference between these treatments is −1.897 [−2.645, −1.134]. We did not find evidence for a silver-spoon-like effect in the germination rate. This experiment allowed us to determine that the observed silver-spoon effect is due to differences in growth and not in germination.

Neurospora crassa produces around 11 times fewer conidia when sucrose concentration is 0.015% (Figure S3), the difference for scaled data was 1.743 [1.519, 1.974]. It is known that number of germinating conidia affects the rate at which the mycelium develops (Richard et al., 2012). Therefore, we always counted conidia and plated the same number of conidia on plates. To make sure that differences in conidial viability or dormancy induced by the different treatments were not a factor, we measured the number of colony forming units in our samples by plating. We did not observe any differences in conidial viability in any generation for conidia coming from either 1.5% or 0.015% sucrose, difference in F_2 was −0.174 [−0.712, 0.379], and in F_3: 0.209 [−0.808, 0.419] (Figures 1c and 2c). Therefore, there must be some qualitative difference in the conidia originating from 1.5% and 0.015% sucrose. We did not observe any differences in spore size: difference in size in F_2 samples was −0.019 [−0.372, 0.305], and for F_3 samples −0.423 [−1.103, 0.225] (Figures 1d and 2d).

We also screened alternative carbon sources for the presence of parental effects. We performed the same experiment but compared the 1.5% sucrose environment against 1.5% arabinose, cellulose, lactose, maltose or xylose. We found a similar silver-spoon effect when *Neurospora* was grown with arabinose, cellulose or lactose. Difference in initial colony size when the strain was grown in sucrose versus arabinose in F_1 was 1.841 [1.198, 2.462]; for cellulose difference was 1.496 [0.973, 2.056]; and for lactose 1.809 [1.159, 2.451] (Figure S4). In each of these environments we observed that the fungus grew always bigger when it experienced sucrose during the previous generation. When comparing sucrose to maltose or xylose we did not observe any parental effects (Figure S4).

3.2 | Transmission and duration of parental effects

We investigated whether the observed parental effects was transgenerational by continuing the experiment to F_3 (Figure 2a). The silver-spoon effect observed in the F_2 did not carry on to subsequent generations. The F_1 environment did not have an effect on initial growth in F_3, the effect of F_1 environment in 1.5% sucrose was 0.121 [−0.122, 0.390] and −0.240 [−0.499, 0.011]in 0.015% sucrose.

We repeated the experiment five times, as in the F_2 experiment we observed some variation in experimental outcomes. In some of the experiments, there appears to a significant F_1 effect in cultures with 0.015% sucrose. However, when all experiments were combined the effect of the F_1 environment overlapped with zero (Figure 2b,f).

We further investigated the intragenerational duration of the silver-spoon effect by looking at the growth rate of the mycelium on F_2 plates in more detail. We had taken three measurements of the colony size on the F_2 plates. When we calculated growth rates instead of using initial colony size, we observed that F_1 environment only had an effect on the growth rate calculated from first time points, and no effect on growth rate in the subsequent time points (Figure S5). Taken together, these experiments suggest that the observed parental effect is an intergenerational effect that matters in the establishment of the mycelium. As the mycelium grows in size, the effect disappears.

3.3 | Fitness consequences of parental effects

To understand the biological significance of the observed silver-spoon effect, we investigated how the parental environment contributes to offspring fitness. We performed the match/mismatch experiment as before, but instead of plating the conidia, we combined the conidia from two strains and let them compete (Figure S1A). We found that the relative fitness of a strain that experienced 1.5% sucrose environment in the previous generation was approximately four times higher when competing against a strain that experienced 0.015% sucrose in the previous generation, in both 1.5% and 0.015% sucrose competition environments (Table 1). This suggests that the small increase in initial speed of colony establishment matters greatly for fitness.

3.4 | Mechanisms of parental effects

To explore the possible mechanisms for the observed parental effects. We investigated nutrient composition, mRNA content of conidia and possible epigenetic effects.

3.4.1 | Protein content and carbohydrate reserves

We quantified protein, glycogen and glucose content in the F_2 conidia grown in either 1.5% or 0.015% sucrose. We observed no difference in the total protein content between treatments, scaled difference was −0.187 [−0.701, 0.311](Figure 3a). However, we found that spores originating from 1.5% sucrose had a higher amount of glycogen, scaled difference was 1.59 [1.009, 2.206]; and a higher amount of glucose, scaled difference was 1.794 [1.44, 2.139] (Figure 3b,c). This suggest that carbohydrate storage in conidia may be responsible for the silver-spoon effect.

3.4.2 | RNA-seq of conidia

To further understand the physiological changes in conidia originating from 1.5% or 0.015% sucrose, we sequenced conidial mRNAs using 150 bp paired end reads. On average, we obtained 13×10^6 reads per library (Table S3). More than 93% of reads in all the samples successfully mapped the reference genome (Table S3). Grouping samples by PCA showed that PC1 represented variation between the sucrose environments, and explained 63.9% of the variation, while PC2 represented variation across samples of the same treatment, and explained 12.4% of the variation (Figure 4a). We also observed a symmetrical distribution of differential gene expression where 6564 (p-adjusted <.01) of the 8925 annotated genes were differentially expressed between treatments (Figure 4b). The p-value distribution obtained from DESEq2 analysis is shown in Figure S6D.

We performed two different enrichment analyses: an over-representation of analysis of KEGG pathways, and a gene set enrichment analysis. Even though the two types of enrichment analysis show slight differences, all of the enriched pathways fall into three categories: metabolism, genetic information and processing, and cellular processes (Figure 4d,e). The vast majority of enriched pathways are metabolic pathways, particularly those involved in the carbohydrate metabolism, while just few of them are involved on other metabolic processes such as lipid, energy or amino acid metabolism. Pathways involved in genetic information processing were: RNA polymerase, ribosome, and proteosome. These pathways are crucial for transcription, translation and protein folding sorting and degradation, respectively. Finally, the peroxisome was the only pathway enriched involved in cellular processes, particularly in transport and catabolism (Figure 4d,e). We also observed that the carbohydrate related pathways, along with proteosome, peroxisome and fatty acid degradation were suppressed in conidia coming from high sucrose environment. We also explored the occurrence of alternative

TABLE 1 Relative fitness effects estimated from competition experiments.

	W_{ij} [95%HPDI]		
Effect	**Combined**	**F_2 1.5% sucrose**	**F2 0.015% sucrose**
*csr-1**	0.58 [0.5, 0.67]	0.51 [0.4, 0.63]	0.66 [0.55, 0.81]
mat A	0.83 [0.72, 0.94]	0.86 [0.7, 1.06]	0.79 [0.65, 0.96]
F_1 1.5% sucrose	4.13 [3.41, 5.14]	4.72 [3.52, 6.52]	3.62 [2.82, 4.88]

Note: Values below 1 indicate that fitness is decreased relative to the other genotype or parental environment, while values above 1 indicate higher relative fitness. The fitness effect of *csr-1** is relative to wild type allele, *mat A* relative to *mat a* and F_1 1.5% is relative to 0.015% sucrose in the parental environment.

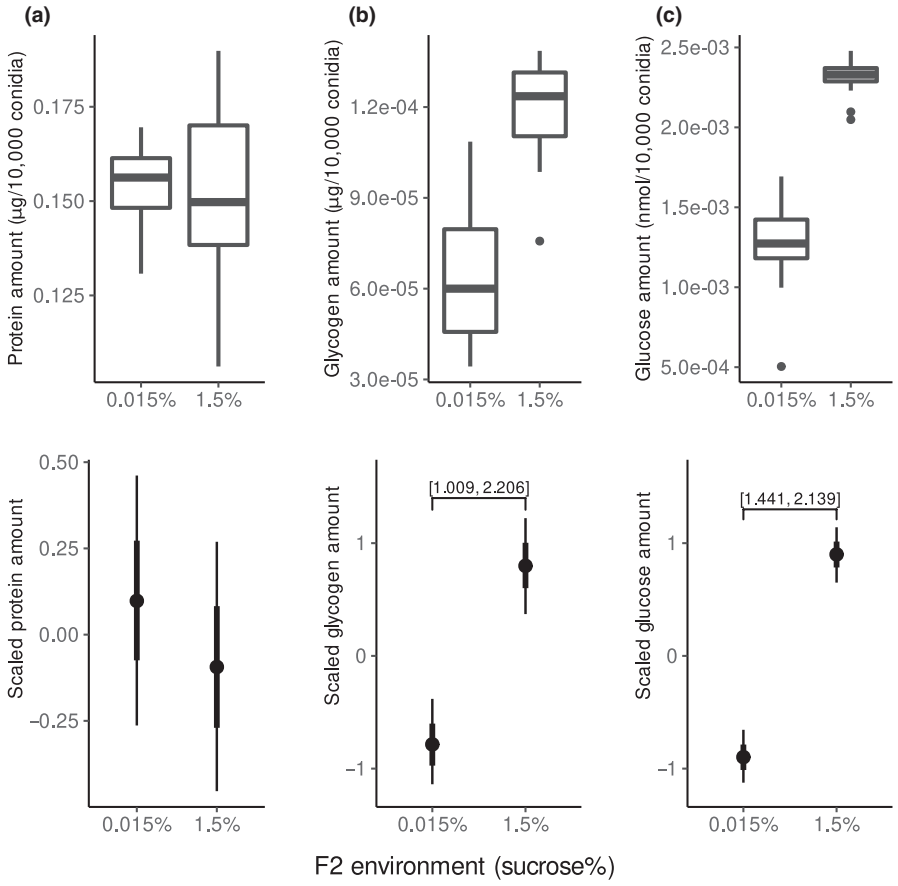

FIGURE 3 Effect of F_1 environment on total amount of protein, glycogen and glucose in conidia. Raw data (top) and the model estimates (bottom) of the total amount of (a) protein (b) glycogen and (c) glucose in conidia originating from 1.5% or 0.015% sucrose. The data in the bottom row are scaled, numbers inside square brackets show the 95% HPDI of the difference between treatments.

splicing events and found 32 cases in total, of which only 17 were in annotated genes. Due to the small number of annotated genes, enrichment analysis of alternatively spliced sites was not possible (see Methods S1 and Table S6).

3.4.3 | Epigenetic mechanisms

To explore whether epigenetic processes could be involved in the mechanism behind the observed silver-spoon effect, we repeated the basic match/mismatch experiment using three mutant lines: Δ*dim-2*, which is deficient in DNA methylation; Δ*qde-2*, which is deficient in small RNA processing; and Δ*set-7*, which is deficient in histone 3 lysine 27 trimethylation. All three strains showed the silver-spoon effect; initial colony size was bigger when the fungus had experienced 1.5% sucrose in the previous generation (Figure 5; Δ*dim-2* = 1.486 [1.125, 1.805]; Δ*qde-2* = 1.658 [1.401, 1.906]; Δ*set-7* = 1.148 [0.743, 1.558]). This suggests that the silver-spoon effect is not based on any of these epigenetic mechanisms.

To further understand the role of epigenetics in the silver-spoon effect, we examined in which domains the 379 genes that belonged to the GSEA-enriched pathways were located. We observed that all genes belonging to the main GSEA pathways, were located in euchromatic regions that were associated with H3K36me2. Twenty genes in total overlapped with H3K27me3 domains, 16 overlapped completely, and 4 genes had partial overlap. Fourteen genes overlapped with H3K9me3, of which only one completely overlapped H3K9me3 (Figure 4c). No genes belonging to the enriched pathways exclusively overlapped heterochromatic regions.

4 | DISCUSSION

Parental effects are a potential mechanism by which organism can deal with environmental challenges (Auge et al., 2017; Badyaev & Uller, 2009; Jensen et al., 2014; Nettle & Bateson, 2015). We have shown that a silver-spoon effect exists in *N. crassa* in response to environments rich or poor in a preferred carbon source. The silver-spoon effect greatly increases competitive fitness, and is likely mediated by carbohydrate storage and gene expression changes in the conidia. So far, to our knowledge, there is only one published investigation on parental effects in fungi (Zimmerman et al., 2016) where maternal investment during sexual cycle of *N. crassa* was explored. Our study is the first in depth investigation into phenotypic, mechanism, and fitness consequences of environmentally induced parental effects in fungi.

In filamentous fungi, the concepts of "individual" and "generation" are somewhat ambiguous, and as a consequence fitness can be difficult to define (Pringle & Taylor, 2002). In *N. crassa* all parts of the mycelium are capable of forming conidia producing structures, so there is no defined germ line. In this study we considered the completion of the asexual life cycle (i. e. from an asexual spore to

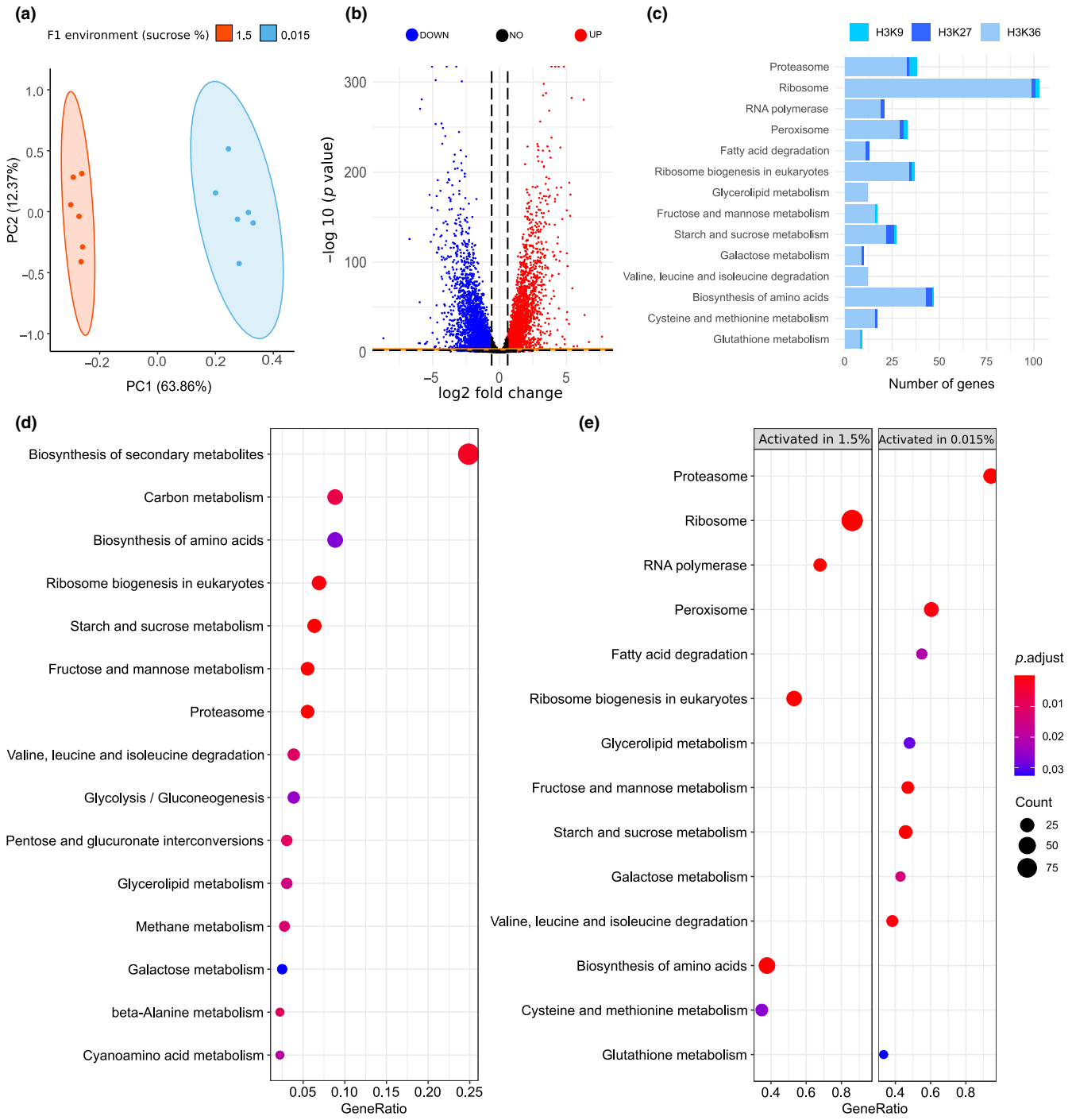

FIGURE 4 DE and enrichment results. (a) Principal component analysis of the read count data. (b) Volcano plot shows the distribution of the DE genes between treatments. Blue dots are downregulated genes, red dots are upregulated genes, and black dots are genes that were not differentially expressed. The horizontal dashed line and the solid orange line indicates the *p*-value of .01 and .001 respectively after correcting for multiple testing, the vertical dashed lines represent log fold changes of 1.5. (c) Number of genes of the most enriched pathways associated to three different histone modification domains: H3K9me3, H3K27me3 and H3K36me2. (d) KEGG enrichment pathways from over-representation analysis (ORA). (e). KEGG enrichment pathways results with gene set enrichment analysis (GSEA). The colour gradient shows the *p*-value and dot size the count of genes in each pathway.

an asexual spore) to be a single generation, as conidia are a distinct dispersal stage in the life cycle of the fungus. Moreover, in certain models of filamentous fungus life-history strategies, spore production is a critical fitness component (Gilchrist et al., 2006).

Evolutionary models state that anticipatory effects can evolve when the environmental fluctuations span from a few generations to tens of generations and fluctuations are at least moderately predictable, but perfect prediction is not required (Kronholm, 2022).

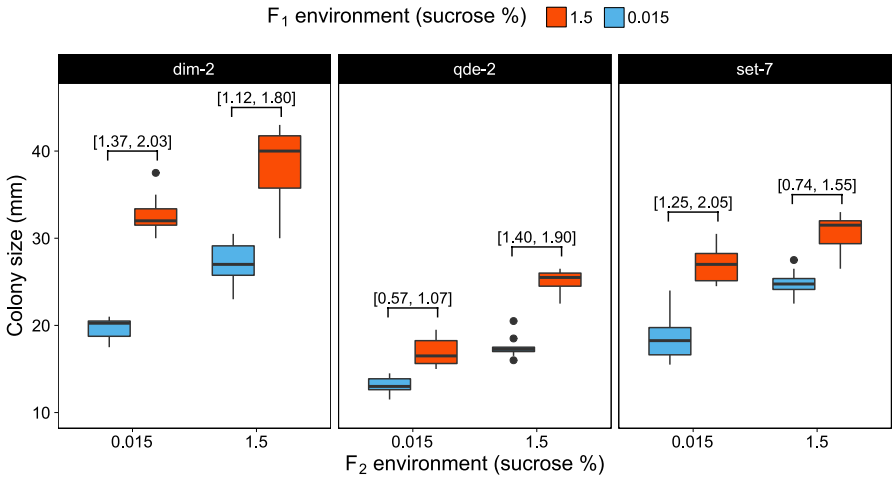

FIGURE 5 Effect of F_1 sucrose concentration on deletion mutant strains. Raw data of initial colony size for three mutant strains: Δ*dim-2*, Δ*qde-2* and Δ*set-7*. Numbers inside square brackets are the 95% HPDI of the differences between treatments obtained from model S1 for scaled data.

Conidia of *N. crassa* are dispersed by wind, so the correlation between the parent and offspring environment is potentially very low. Thus, it could be argued that absence of anticipatory effects is unsurprising. However, even if the theoretical maximum dispersal distance can be potentially hundreds of kilometres, actual dispersal distances experienced by majority of the spores can be much lower. The spores of some fungal species tend to disperse in clumps, although this impedes the spore travel distance, it stimulates germination and protects the spores from environmental challenges (Golan & Pringle, 2017). There are no estimates of dispersal distance or neighbourhood size for *N. crassa* in the wild, so we cannot know for certain what is the potential for evolution of anticipatory effects.

Silver-spoon effects are those fitness advantages that an organism may have, because its parents had access to abundant resources (Bonduriansky & Crean, 2018; Pigeon et al., 2019; Spagopoulou et al., 2020). In this case, the fungus in favourable environments had access resources that it invested in the next generation (i.e. spores). We demonstrated that even if the silver-spoon effect was only relevant during initial growth, it increased fitness. We measured the fitness effects of the observed silver-spoon effect in competition with other spores. So, it could be that the silver-spoon effect is beneficial only if spore density in the environment is high, like in the inoculation spot in our experiment. When conidia of *N. crassa* germinate they can fuse with each other which increases the fitness of the mycelium (Richard et al., 2012). However, only hyphae that are of the same mating type and have compatible allorecognition loci can fuse, as fusion of incompatible hyphae results in cell death (Bastiaans et al., 2015). We observed the silver-spoon effect both when there was no competition, and when the spores had to compete with an incompatible genotype. It has been shown that competition of incompatible hyphae results in effects that are independent of spore density (Bastiaans et al., 2015), and it is possible that the silver-spoon effect may interact with these competitive interactions possibly amplifying the fitness benefit of the silver-spoon effect. If a single spore lands in a patch on its own and has no competitors, then effects on early growth may not matter. However, if the resource patches are ephemeral due to drying out for example, then faster early growth may be beneficial even at low spore densities.

The adaptive value of silver-spoon effects are controversial. Silver-spoon effects have been classified by some authors as non-adaptive (Marshall & Uller, 2007) because, unlike anticipatory effects, silver-spoon effects do not provide and adaptive match of the offspring phenotype to the environment. However, Bonduriansky and Crean (2018) argued that silver-spoon effects can still considered to be adaptive as they can enhance parental fitness by increasing the performance of the next generation. If the mechanism of the silver-spoon effect is not completely passive, but requires some action on the part of the parent, such as nutrient allocation and so forth, it is conceivable that some genotypes would be unable to allocate resources to the offspring even if those resources would be available. To investigate if the silver-spoon effect described here is an inevitable consequence of the parental environment, we would need to establish if strains with different genetic backgrounds differ on their efficiency of parental resource transfer. If selection favours traits that increase investment in offspring, such as storage of metabolic resources and efficiency of cellular processes, this can lead to the evolution of a variety of strategies for parental investment (Bonduriansky & Crean, 2018).

Silver-spoon effects based on differential investment could influence adaptation by indirectly affecting allele frequencies through altered population dynamics (Hopwood et al., 2014; Plaistow et al., 2006), much like phenotypic plasticity can keep population size high in the face of environmental change, and influence adaptation indirectly via higher mutational supply (Chevin et al., 2010). For example Benton et al. (2005) showed that differential maternal investment, in the mite *Sancassania berlesei*, influenced population dynamics for more than one generation. First, maternal investment influenced growth rate, and therefore age and size at maturity which affected egg size on the next generation. Second, maternal allocation also indirectly affect population dynamics by changing the competitive environment of the offspring which in turn influenced, growth rate, fecundity and age of maturation (Benton et al., 2005). Also, it has been shown that carry over effects in bees can significantly decrease population growth putting at risk population persistence (Stuligross & Williams, 2021).

To understand the scope that silver-spoon effects can have in natural populations, it is crucial to understand their mechanisms. Glycogen serves as a carbon and energy reserve, and glucose as the main energy source in *N. crassa* (Bertolini et al., 2012; Virgilio et al., 2017; Wang et al., 2017). Cultures that were grown under low sucrose conditions were limited by the amount of glucose in the medium, as spore production was severely limited. In addition, the spores produced by a mycelium in 0.015% sucrose had lower glycogen and glucose levels. Glycogen storage and glucose availability in the spores gives a fitness advantage to the fungus, even if in the next generation it grows in a low sucrose environment.

In conjunction with sugar content in the spores, we found that the silver-spoon effect involved a dramatic gene expression change, in which pathways related to sugars and carbohydrate metabolism were over-expressed in conidia that experienced 0.015% sucrose. These results are explained by the carbon catabolite repression, a common process among fungi, where the production of enzymes responsible for degrading plant cell wall material is inhibited while preferred carbon sources (e.g. sucrose), are available in the environment.

In nature, *N. crassa* grows on dead plant material, and thus, it relies on breaking down the plant biomass components (Benz et al., 2014; Huberman et al., 2017; Sun et al., 2012). For this reason, *N. crassa* has a vast enzymatic toolkit that allows it to utilize the variety of simple or complex carbon sources present in the plant cell wall. However, it would be disadvantageous to produce enzymes to break down nutrients that are not available in the substrate (Huberman et al., 2017). To avoid such costs, *N.crassa* has evolved systems to accurately detect the nutrients available in the environment to produce only the needed enzymes (Huberman et al., 2017; Sun & Glass, 2011; Temporini et al., 2004). When sucrose is present, the carbon catabolite repression silences the expression of lignocellulolytic genes (Huberman et al., 2017). When sucrose is not available, the carbon catabolite repression is diminished causing elevated levels of lignocellulolytic genes expression allowing a small secretion of a vast number of different enzymes that allow the fungus to utilize alternative carbon sources (Sun & Glass, 2011). This produces gene expression patterns in which the fungus expresses ribosomal proteins and functional categories related with primary metabolism pathways in sucrose rich environments, while under glucose starvation fungus expresses sugar and carbohydrate metabolism related pathways (Benz et al., 2014; Xie et al., 2004). This metabolic behaviour has been previously observed in *N. crassa* and other fungal species (New et al., 2014). The mycelium of the next generation will directly germinate from the conidia, therefore the mRNA content of the spores impacts the performance of the next generation.

Similar silver-spoon effects were also present when *N. crassa* grew on media containing arabinose, cellulose, or lactose, and absent when it grew on maltose and xylose media. A possible explanation for this might be that the first three environments represent a disadvantage over the sucrose environment and they will trigger the carbon catabolite repression. For example, although cellulose is one of the main plant cell wall components, it is very difficult to degrade, lactose is slowly metabolized (Comp & Lester, 1971; Lester et al., 1962), and arabinose rewires the fungal cell metabolic pathway triggering a similar response to carbon starvation conditions (Li et al., 2014). On the contrary, maltose and xylose are not very challenging environments, xylose is one of the preferred carbon sources (Sun & Glass, 2011) and maltose is actually commonly used as a banding media when studying circadian rhythms (Martens & Sargent, 1974).

We observed that strains deficient in different epigenetic mechanisms did not prevent silver-spoon effects from occurring. In *N.crassa* dim-2 and set-7 activity is related with DNA methylation and H3K27 trimethylation respectively. These epigenetic marks are associated with heterochromatic regions in *N. crassa*, which have low gene density and expression levels (Gessaman & Selker, 2017; Jamieson et al., 2013). Most genes belonging to the pathways showing differential expression were associated with euchromatic regions. It appears that carbohydrate metabolism is not under strict epigenetic control in *N. crassa*. The quelling deficient element gene (*Qde-2*) did not interfered with the occurrence of the silver spoon effects neither. *Qde-2* codes an argonaute protein that is involved in post-transcriptional gene silencing also known as quelling Dang et al. (2011). However, so far there is no evidence showing that post-transcriptional gene silencing is a regulation strategy in response to glucose starvation environments in filamentous fungi.

Finally, we want to stress the importance of expanding the taxonomic representation on parental effects research and to investigate their adaptive potential even if they are short-lived. In comparison to anticipatory effects, silver-spoon effects have been widely overlooked even that some of their aspects suggest they might the most widespread type of parental effect across taxa (Bonduriansky & Crean, 2018). Contrary to anticipatory effects, silver-spoon effects do not depend on the environment predictability nor on complex mechanisms to asses the environment and adjust the offspring phenotype accordingly. Silver-spoon effects may influence the ecology and evolutionary processes in several eukaryotes across the tree of life.

AUTHOR CONTRIBUTIONS

I.K. and M.V. conceived the study. M. V., P.A.M.S., N. N. M. and I.K. performed experiments. M.V., I.K., M.V. and P.A.M.S. analysed the data. M.V and I.K. wrote the manuscript. All authors edited the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

RNA-sequencing data have been deposited to the short read archive, project number PRJNA907747, with sequence accession numbers SRX18465547–SRX18465558. Other data and scripts are available at https://github.com/mariana19901990/Neurospora-crassa-Parental-effects.

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SUPPORTING INFORMATION

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