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# Correlation of mycelial growth rate with other phenotypic characters in evolved genotypes of *Aspergillus nidulans*

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## ABSTRACT

Fungal populations can adapt to their environment by the generation and fixation of spontaneous beneficial mutations. In this study we examined whether adaptation, measured as an increased mycelial growth rate, has correlated responses in the filamentous fungus *Aspergillus nidulans* with several other metric characters that could be important fitness components (colony forming units, germination speed, and biomass formation). Studying 60 populations that had evolved over 800 generations by experimental evolution, we find that only mycelial growth rate increased during adaptation to growing on solid medium. We further found that among evolved strains colony forming units is negatively correlated with mycelial growth rate and that colony forming units and biomass formation show a positive correlation. Our results give insight into changes in fungal phenotype as a result of adaptation and suggest that mycelial growth rate is the only available target of selection.

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## Introduction

Adaptation is an evolutionary process that occurs through natural selection acting on genetic variation. Adaptation frequently results in phenotypic divergence and patterns of phenotypic divergence in both nature and the laboratory can therefore provide valuable insight into evolutionary processes. In some instances, observed divergence in a particular trait is well understood (e.g. in artificial selection experiments with prescribed targets of selection) but often – for example, in experimental studies of adaptation, trait evolution is not restricted to traits prescribed by the experimenter. Upon observing adaptive changes, the experimenter would like to know which traits have conferred higher fitness. Similarly,

experimenters often need to use one or a few traits as surrogate measures of (evolutionary) fitness for a variety of purposes. However, when considering phenotypic evolution of multiple characters, it can be difficult to discern the degree to which observed responses in any given trait are the result of selection directly favouring these or merely the result of indirect selection through the correlations among traits (Lande 1979; Lande & Arnold 1983).

Adaptation is potentially limited by the availability of genetic variation in selected traits as well as by correlation among traits. In initially isogenic populations, adaptation can be studied in the laboratory using experimental evolution: large microbial populations are allowed to adapt to higher fitness in their environment by propagating them for prolonged

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periods of time by periodic serial transfer to fresh medium (Elena & Lenski 2003). Here, adaptation relies on the occurrence of spontaneous beneficial mutations (e.g. a shift in the value of a trait that is selectively favourable) that may be accompanied by pleiotropic effects on other traits. While the utility of examining of mean phenotypic change of multiple characters following experimental evolution has been well appreciated, we would like to illustrate the additional utility of examining the trait correlation structure among experimentally diverged lineages. This provides useful insight because the persistence of such correlations can point to both genetic (e.g. pleiotropy) and selective agents (e.g. trade-offs) that may have facilitated or constrained evolutionary trajectories. Similarly, the correlation structure among traits is of interest when trying to identify traits that might be considered surrogate measures of fitness. For example, positive correlations among traits could result from a mutation that increased ability to allocate to all traits and, thus, any of the measured traits would provide a reasonable proxy for fitness. Conversely, if adaptation was associated with a shift in allocation to various traits, this could appear as negative correlations and, clearly, any single trait need not provide an accurate picture of the phenotypic basis for adaptation.

In a recent study, we reported the occurrence and fixation of beneficial mutations leading to higher fitness in *Aspergillus nidulans* populations adapting to a novel environment (Schoustra et al. 2009). In that study, 120 replicate populations derived from a common ancestor were allowed to adapt to novel conditions by repeated serial transfer to fresh medium for 800 mitotic generations. As is common in filamentous fungi such as *A. nidulans* and *Aspergillus niger*, fitness was measured as the mycelial growth rate (MGR), which is the rate of colony expansion over time. This is a metric that is highly repeatable across assays and easy to measure (De Visser et al. 1997; Pringle & Taylor 2002; Bruggeman et al. 2003; Schoustra et al. 2005). Importantly, MGR has been found to correlate well with other possible measures of fitness, such as competitive ability in direct head-to-head competitions – which is regarded as the golden standard for measuring all-inclusive evolutionary fitness (Elena & Lenski 2003) – and spore production over time (de Visser et al. 1997; Pringle & Taylor 2002; Schoustra et al. 2009). The protocol for the selection experiment was as follows. At the start, a droplet of 5 µl containing 500 spores was placed in the centre of a Petri dish containing solid medium for each replicate population. After 5 d of incubation, the resulting fungal colony was inundated with 5 ml of salts solution to wash off fungal material. Dilutions of the resulting suspension – 5 µl containing either 50 000 (large bottleneck) or 500 (small bottleneck) – were used to found the next cycle of the selection experiment. After 800 mitotic generations, populations had reached a wide range of evolutionary fitness optima by fixing 1, 2 or 3 beneficial mutations with fitness (MGR) increases varying from just a few percent to over 100 % as compared to the fitness of the ancestral genotype (Schoustra et al. 2009).

In this present study, we measured the evolutionary changes in three additional characters and their correlation with MGR. Apart from MGR we measured biomass formation (BM), colony forming units (CFU), and fraction of fast germinating spores including spores that are diploid (rather than

haploid) (DPL). These three characters were measured per standardized surface area of mycelium – i.e. not over time – by spreading a spore suspension of the genotype of interest solid medium and letting a mycelium completely cover the entire surface. Given the protocol for the selection experiment described above, we may expect that the number of CFU and the amount of biomass formation could have been under selection as well. We used 60 evolved strains from the selection experiment described above (Schoustra et al. 2009); 30 strains from the large and 30 strains from the small-bottleneck treatment. Since the supply of spontaneous mutations is determined by the effective population size, which in turn is determined by the bottleneck size (Bell 2008), we might expect different outcomes when comparing evolved strains taken from those different bottleneck treatments. Our results give insight in changes in fungal phenotype as correlated response to the evolutionary process of mutation and positive selection on beneficial mutations.

## Materials and methods

### Strains and culture conditions

We used 60 strains of the filamentous fungus *Aspergillus nidulans* derived from an 800-generation selection experiment described in Schoustra et al. (2009). The strain that founded the selection experiment was WG615 (II: wA3; III: fldA1; IV: pyroA4; VIII: veA1). For the present study, we selected 30 large and 30 small bottleneck lineages from this selection experiment, capturing a representative range of final fitness (MGR) achieved. Within groups of both large and small bottleneck, we picked around ten strains that had fixed either one, two or three beneficial mutations. We used solid special Complete Medium (CM) for all experiments, set at pH 5.8, consisting of NaNO<sub>3</sub> 6.0 g l<sup>-1</sup>; KH<sub>2</sub>PO<sub>4</sub> 1.5 g l<sup>-1</sup>; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g l<sup>-1</sup>; NaCl 0.5 g l<sup>-1</sup>; 0.1 ml of a saturated trace element solution containing FeSO<sub>4</sub>, ZnSO<sub>4</sub>, MnCl<sub>2</sub>, and CuSO<sub>4</sub>; tryptone 10 g l<sup>-1</sup>; and yeast extract 5 g l<sup>-1</sup>, agar 10 g l<sup>-1</sup>, and (added after autoclaving) glucose 4.0 g l<sup>-1</sup>. Cultures were incubated at 37 °C.

### Mycelial growth rate (MGR)

MGR was measured in triplicate by placing 5 µl of a dense spore suspension in the centre of a Petri dish with solid CM. After 5 d of incubation, the diameter of the resulting fungal colony was measured in two perpendicular directions. This is a repetition of the assay presented in Schoustra et al. (2009).

### Biomass formation per surface area of mycelium (BM)

30 µl of a dense spore suspension was spread out on a Petri dish with CM in three-fold for each genotype. After 1 week of incubation, all fungal material of every entire plate was scraped off using 5 ml saline–Tween (water containing NaCl 0.8 % and Tween-80 0.05 %), yielding a suspension containing mycelium and spores which was vigorously vortexed for 45 s. Filter papers (Whatman 1001-042; 42.5 mm) were weighed on a fine scale before applying 1 ml of suspension. After 24 h of drying, the filter paper was weighed again, the difference in

weight is attributable to the biomass in the suspension, which in turn is attributable to all biomass formed on the surface of the solid medium in the Petri dish (i.e. this assay does not capture the biomass of any hyphal or other structures that have grown into the agar). All assays of all strains were done in one large assay.

#### Colony forming units per surface area of mycelium (CFU)

From the same suspension as for the biomass formation assay, serial dilutions were made in a minimal salts solution ( $\text{Na}_2\text{HPO}_4$  6.7 g,  $\text{KH}_2\text{PO}_4$  3 g,  $\text{NaCl}$  0.5 g,  $\text{NH}_4\text{Cl}$  1.0 g, 1000 ml  $\text{dH}_2\text{O}$ ) that were plated on solid CM supplemented with triton ( $40 \mu\text{l l}^{-1}$ ). Triton reduces colony size of fungal colonies to facilitate counting. After 48 h of incubation, colonies were counted and the total number of CFU in the original suspension was calculated. Colonies could originate from nuclei in spores or nuclei in viable mycelial fragments.

#### Fraction of fast germinating spores including diploids (DPL)

We estimated the fraction of fast germinating spores including diploid nuclei present in the same suspensions used for the biomass formation assay using the so-called sandwich method (modified from Clutterbuck 1974; Todd et al. 2007). Using the CFU counts above used to calculate CFU, a dilution of the spore suspension was mixed in 3 ml Minimal Medium with pyridoxine (set at pH 5.8, consisting of  $\text{NaNO}_3$   $6.0 \text{ g l}^{-1}$ ;  $\text{KH}_2\text{PO}_4$   $1.5 \text{ g l}^{-1}$ ;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$   $0.5 \text{ g l}^{-1}$ ;  $\text{NaCl}$   $0.5 \text{ g l}^{-1}$ ; 0.1 ml of a saturated trace element solution containing  $\text{FeSO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{MnCl}_2$ , and  $\text{CuSO}_4$ ; agar  $10 \text{ g l}^{-1}$  and (added after autoclaving) glucose  $4.0 \text{ g l}^{-1}$ , and pyridoxine  $0.1 \text{ mg l}^{-1}$ ) and poured out in an empty Petri dish such that  $1 \times 10^4$  spores were present. After solidification, an additional sandwich layer of 35 ml Minimal Medium was poured on top. After 20 h of incubation, colonies were counted (after 48 h the plates were fully overgrown). Since diploids grow substantially faster through the sandwich layer, we expect most if not all colonies present after 20 h to be diploid rather than haploid. For several isolated colonies, we confirmed that they were vegetative diploids and not haploids using CM supplemented with benomyl (Upshall et al. 1977). The DPL assay was done in triplicate for each genotype.

#### Statistical analyses

Analyses were conducted on the trait values averaged from three independent replicates for 30 evolved lineages in each bottleneck treatment. Colony forming units (CFU) and fraction of fast germinating spores including diploids (DPL) were log<sub>e</sub> transformed prior to analyses. Biomass formation (BM) and mycelial growth rate (MGR) were approximately normally distributed and did not require transformation. For each character, we asked if the mean of all evolved genotypes deviates from the ancestor using a t-test. The P-value at which to reject the null hypothesis of no difference was adjusted to 0.0125 to correct for multiple testing. To test for correlations between traits, we first evaluated the bivariate relationships among traits for data pooled across treatments, using Pearson's product moment correlation and the associated pair-wise

significance. The Pearson's product has a value between  $-1$  and  $1$ , whereby values close to  $0$  are suggestive of no correlation between the two characters and values near  $-1$  and  $1$  are suggestive of a very strong association. A negative or positive value is indicative of a positive or negative slope of the relationship. We also conducted these analyses separately by treatment to account for possible differences due to the bottleneck procedure. To formally examine possible treatment-specific bivariate relationships, we used an ANCOVA whereby one trait was modelled as a function of the other, while including treatment and treatment  $\times$  trait interaction terms as predictor variables. Statistical analyses and significance testing performed using JMP<sup>®</sup> v.5.0.1a (SAS Institute, NC).

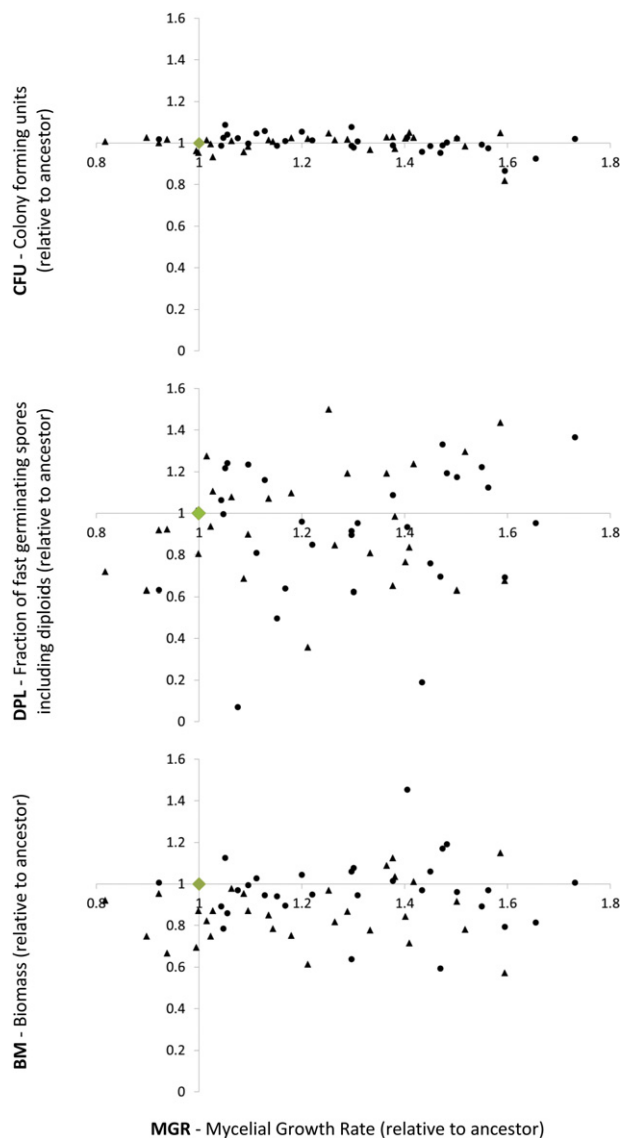
## Results

Fig 1 shows the response to selection of three characters as a function of change in MGR, expressed relative to the ancestor (i.e. the trait value of the ancestor is set at 1). The average value for MGR for evolved strains is 1.25, indicating a 25 % increase relative to the ancestor, which is statistically significant (one-sided t-test;  $t_{59} = 9.07$ ,  $P < 0.0001$ ). For CFU, the average value for the evolved strains relative to the ancestor is 1.0019, which is not statistically different from 1, indicating no change relative to ancestor ( $t_{59} = 0.328$ ,  $P = 0.74$ ). For DPL the average value for the evolved strains relative to the ancestor is 0.944, suggesting a 5 % decrease relative to the ancestor, however this decrease is not statistically significant ( $t_{59} = -1.44$ ,  $P = 0.16$ ). For BM the average value for the evolved strains is 0.91, indicating a 9 % decrease relative to the ancestor, which is statistically significant ( $t_{59} = -4.13$ ,  $P = 0.0001$ ). Comparing the two effective population sizes, there is no statistically significant difference for MGR, CFU, and DPL, but there is a difference for BM (ANOVA: MGR  $F_{1,58} = 3.09$ ,  $P = 0.08$ ; CFU  $F_{1,58} = 0.11$ ,  $P = 0.73$ ; DPL  $F_{1,58} = 0.56$ ,  $P = 0.45$ ; BM  $F_{1,58} = 7.58$ ,  $P = 0.0078$ ). For BM, the strains with the small bottleneck decreased less than for the large effective population size (to 0.97 for the small effective population size and 0.86 of the large; only the latter being significantly different from 1).

Fig 2 shows the pair-wise relationships between all characters measured, with different symbols for strains that had evolved with the large (triangles) and the small (circles) bottleneck treatment (Schoustra et al. 2009). Table 1 lists the statistical characteristics of these relationships. CFU and MGR exhibited an overall negative correlation (pooled:  $r = -0.25$ ,  $p = 0.052$ , Fig 1) though the relationships appeared to differ in two treatments (treatment  $\times$  trait interaction: ANCOVA,  $F_{1,1} = 2.97$ ,  $p = 0.09$ ); most likely the result of a strong negative correlation in small-bottleneck treatment ( $r = -0.49$ ,  $p = 0.006$ ) and only a weak relationship in the large-bottleneck treatment ( $r = -0.06$ ,  $p = 0.237$ ). We did not detect a significant correlation between MGR and BM and between MGR and DPL.

Considering all pair-wise correlations between all the four measured characters (Fig 2; Table 1), the only pair-wise correlation that was statistically significant in both the large and small-bottleneck treatments was that between BM and CFU ( $r = 0.38$ ,  $p < 0.04$  for both large and small bottlenecks;  $p = 0.003$  when pooling data). Similarly, CFU and DPL were positively related when considering pooled data ( $r = 0.26$ ,





**Fig 1 – Response to selection of CFU, fast germinating spores including diploids (DPL), and biomass formation (BM) shown as a function of MGR. Triangles are evolved genotypes of the large-bottleneck treatment, circles are evolved genotypes of the small-bottleneck treatment. The green diamond shows the ancestor of the evolution experiment. All values shown are expressed relative to the value of the ancestor of the 800-generation selection experiment: a value of 1 shows no change, values above 1 show an increase and values below 1 show a decrease relative to the ancestor.**

$P = 0.048$ , Fig 2), though the treatment-specific correlations were not significant (small bottleneck:  $r = 0.30$ ,  $p = 0.103$ ; large bottleneck:  $r = 0.22$ ,  $p = 0.237$ ).

## Discussion

In this study we investigated responses to selection that had occurred during long term evolution of *Aspergillus nidulans*

**Table 1 – Pair-wise correlations between the four characters. Datasets are shown separately of the strains that evolved with the small bottleneck, the large bottleneck, and all pooled data. The numbers show Pearson's correlation coefficient ( $r$ ), numbers in brackets show pair-wise  $p$ -values, statistically significant values are in italics (see Methods).**

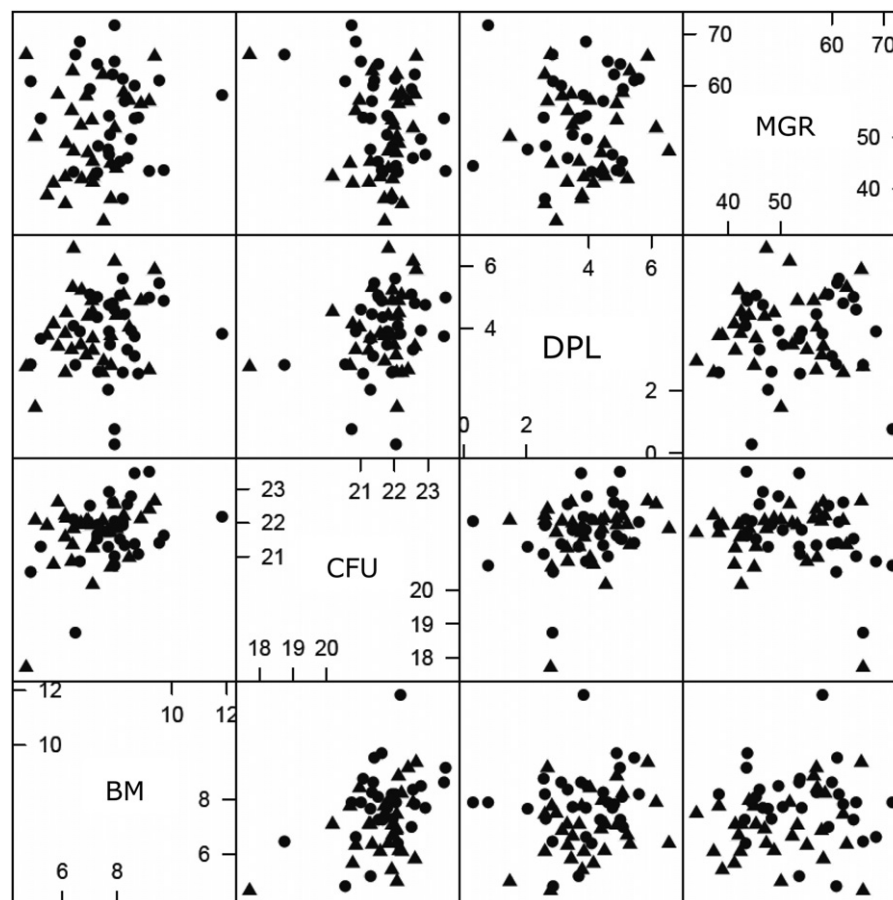
	BM	CFU	DPL
Small $n = 30$			
MGR	–0.14 (0.463)	–0.49 (0.006)	0.04 (0.837)
DPL	0.16 (0.401)	0.30 (0.103)	
CFU	0.38 (0.039)		
Large $n = 30$			
MGR	0.18 (0.329)	–0.06 (0.741)	0.11 (0.555)
DPL	0.31 (0.095)	0.22 (0.237)	
CFU	0.38 (0.038)		
Pooled $n = 60$			
MGR	0.09 (0.500)	–0.25 (0.052)	0.05 (0.701)
DPL	0.18 (0.170)	0.26 (0.048)	
CFU	0.38 (0.003)		

BM: biomass formation per surface area; CFU: colony forming units per surface area; DPL: fast germinating spores including diploids; MGR: mycelial growth rate.

strains by measuring four characters: MGR (fitness), biomass formation (BM), CFU, and fraction fast germinating spores including diploid nuclei (DPL). BM, CFU, and DPL were measured per surface area of mycelium. We found that only MGR on average increased in response to selection, that CFU and DPL did not change on average and that BM decreased. Apart from the response to selection by comparing trait values of evolved strains with those of the ancestor of the evolution experiment, we looked for correlated responses among the four characters in the evolved strains. We found a negative correlation between MGR and CFU and a positive correlation between CFU and BM.

During the 800-generation experimental evolution study from which the genotypes used in this study were derived, selection was suspected to directly target MGR, since this trait has been shown to be a good proxy for evolutionary fitness (Pringle & Taylor 2002; Schoustra et al. 2009). As can be seen from the large range of MGR values in the assay (Fig 1), evolved strains varied considerably in MGR after 800 generations of evolution, but on average increased by around 25 %. Considering the protocol of the selection experiment, which involves the washing off of spores from the surface of the colony, we do not necessarily expect only the expansion rate of mycelium to increase through selection. Alternatively, mutations that cause a more dense coverage of spores on the surface of the colony (measured as CFU) and associated biomass formation (BM) could also have fixed during the evolution experiment. This was not the case. A likely explanation is that mutations that increase spore density or biomass formation are not available.

This is further illustrated by the negative relation (trade-off) between MGR and CFU, which implies that evolved strains with higher MGR on average are investing more in producing mycelium and associated more rapid colony expansion rather than in the production of more spores per surface area. Our



**Fig 2** – Scatter plots of all characters measured. BM: biomass formation (mg) per ml spore and mycelium fragment suspension; CFU: colony forming units per ml spore and mycelium suspension ( $\log_e$  transformed counts); DPL: fraction of fast growing nuclei including diploid nuclei present in the suspension ( $\log_e$  transformed); MGR: mycelial growth measured as colony diameter after 5 d of growth (mm). Different symbols represent data points for strains taken from the large (triangles) and small (circles) bottleneck treatment that was applied during the selection experiment (Schoustra *et al.* 2009). Table 1 lists statistical parameters. The values of the ancestral strain that founded the selection experiment are BM: 8.1 mg; CFU: 21.88; DPL: 4.8; MGR: 41.4 mm.

results thus suggest that the relative investment in spore production may have declined with higher mycelial expansion rate which is reflected in MGR. In addition, the absence of a relationship between BM and MGR suggests that colonies that expand at a higher rate do not just make less dense mycelium and/or divert more resources into rapidly covering a surface. Mycelial hyphae make up most of the biomass in a colony, with the cell walls of hyphae alone contributing to one-quarter of the total biomass (De Groot *et al.* 2009; El-Ganiny 2011). The fact that CFU and BM are positively correlated is consistent with the interpretation that more nuclei need more biomass. The biomass formation assay was performed using hyphal colonies that had grown out on the surface of solid agar. Alternatively, we could have used the method of growing grow strains in liquid culture and to then directly measure all biomass produced by filtering. We chose not to take this approach since the fungal strains used in this study had evolved on solid agar for 800 generations. An assay of biomass formation using solid medium more closely resembles the selection

environment, maximizing the possibility of detecting changes that are the result of selection.

The fraction of fast germinating spores including diploids (DPL) did not correlate in any way with MGR, although there is a positive correlation between DPL and CFU. Apparently, faster germination does not contribute to higher MGR. Most fast germination spores are expected to be diploids (see Materials and methods). The formation of diploids is the start of the parasexual cycle (Pontecorvo 1956), apparently there is no higher or lower tendency to initiate in this cycle depending on MGR. In a previous study (Schoustra *et al.* 2010), we showed that investment in sexual reproduction, as measured by the production of cleistothecia, is higher when fitness (MGR) is lower, a phenomenon called fitness associated sex (Agrawal 2006; Hadany & Otto 2009; Otto 2009). The results of the present study suggest fitness associated sex does not apply to the parasexual cycle, a cycle that could also generate novel genetic variation by mitotic recombination and/or recombination between

whole chromosomes. We should however caution against overinterpretation of the DPL results since our method, which relies on diploids growing faster through a layer of agar than haploids, may have not exclusively detected diploids. Whereas we confirmed the ploidy of several colonies that appeared on the sandwich plates, some may actually be fast growing haploid types, which would indicate variation for haploid germination time and/or hyphal expansion rate through solid medium.

We find differences between the strains that had evolved under the different bottleneck treatments, especially for the relationship between MGR and CFU. The bottleneck applied during serial transfer in a selection experiment determines the effective population size (Bell 2008), which in turn determines the supply of mutations (De Visser *et al.* 1999). The small and large bottleneck populations potentially sampled a different set of (beneficial) mutations, where large bottleneck populations may have sampled a larger range (Handel & Rozen 2009). This possibility is subject of further study.

For laboratory evolutionary studies it is essential to readily and reliably measure fitness of ancestral and evolved genotypes (Elena & Lenski 2003). The gold standard for measuring fitness in evolutionary studies is by direct head-to-head competitions between two or more genotypes (Elena & Lenski 2003; Jasmin & Kassen 2007). However, direct competitions are labour intensive and can have considerable noise. In filamentous fungi such as *A. nidulans* and *Aspergillus niger*, fitness is usually measured as MGR – the rate of colony expansion over time. This is a metric that is easy to measure and highly repeatable across assays. Importantly, MGR has been found to correlate well (and statistically significantly) with competitive ability in direct head-to-head competitions (Schoustra *et al.* 2009) and also with spore production over time (De Visser *et al.* 1997; Pringle & Taylor 2002). The results of the present study indicate that the surface area based characters CFU and BM, though tightly correlated with one another, cannot be used as surrogate fitness measure. Collectively, these results caution against the reliance on a single trait to necessarily provide an adequate summary measure of fitness, such as carrying capacity or exponential growth rate. In fact, some of the traits considered here are likely to comprise only components of total fitness, some of which might exhibit trade-offs (Stearns 1992; Fitzsimmons *et al.* 2010). The difficulty in measuring total fitness is a nontrivial endeavour that is well appreciated, however, we feel that in many cases, it is more useful to define a measure of fitness based on biological relevance or according to the measures appropriate to the study.

In summary, our results revealed that during adaptation of *A. nidulans* strains to novel conditions on solid growth medium the MGR is the main target responding to selection. This suggests that, under the conditions used during the experimental evolution study, increased spore production or faster germination were not targets of direct selection or could indicate a lack of available mutations to evolutionarily alter this trait. One possibility is that spore production and germination have long been under selective pressures allowing fixation of all possible beneficial mutations that could evolutionarily optimize this trait in the wild before this fungal

strain was brought into the laboratory rendering those traits unavailable to selection.

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