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Scaling Fitness Optima-Studying Adaptive Evolution With Multiple Genotypes of *Aspergillus Nidulans*

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Scaling fitness optima–Studying adaptive evolution with multiple genotypes of *Aspergillus nidulans*

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If I have seen a little further it is by standing on the shoulders of Giants
—Isaac Newton

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Danna Gifford
Ottawa, December 2010

Statement of contributions

Chapter 2 Laboratory work was completed with assistance from S E Schoustra

Chapter 3 The empirical work in this chapter was designed and executed in collaboration with S E Schoustra

NB Chapters 2 and 3 of this thesis are manuscripts in preparation for submission to journals As such, some of the introductory content may overlap

Abstract

Adaptation involves the successive substitution of beneficial mutations by selection, a process known as an adaptive walk Theoretical models of adaptation have made predictions regarding the properties of adaptive walks, but we know little empirically about how adaptation differs between different genotypes faced with the same environment, knowledge that is critical to a broad understanding of natural adaptation Furthermore, as the body of theoretical knowledge continues to grow, it is becoming apparent that the conventional model systems used to study adaptation, chiefly single-celled organisms, are not well-suited for studying adaptation in spatially structured environments, and, barring yeasts, cannot be used to study the effects of sex on adaptation This thesis tackles both questions using the fungus *Aspergillus nidulans*, first demonstrating that the selection coefficients of mutations, but not the total number, are affected by initial adaptedness to an environment and then developing a mathematical growth model that will allow for further development of theoretical models of adaptation in spatially structured environments

Résumé

L'adaptation implique l'accumulation successive de mutations avantageuses par la sélection naturelle, un processus connu sous le nom de « chemin adaptatif » Les modèles théoriques de l'adaptation ont émit des prédictions concernant les propriétés des chemins adaptatifs mais empiriquement, nous ne savons que très peu la façon dont l'adaptation diffère entre différents génotypes vivant dans un même environnement, aspect critique pour une compréhension globale de l'adaptation naturelle De plus, grâce à l'augmentation de nos connaissances théoriques, il devient clair que les systèmes modèles habituellement utilisés dans l'étude de l'adaptation, principalement des organismes unicellulaires, ne sont que peu adapté à l'étude de l'adaptation dans des environnements spatialement structurés Mis à part dans le cas de la levure, ces systèmes ne peuvent être utilisés pour étudier les effets du sexe sur l'adaptation Cette thèse aborde ces deux questions par des expériences utilisant le moisissure *Aspergillus nidulans* Tout d'abord, j'ai démontré que la valeur sélective des mutations, mais pas leur nombre total, est influencée par la valeur sélective initiale J'ai ensuite développé un modèle de croissance mathématique qui permettra des développements futur de modèles théoriques de l'adaptation dans des environnements spatialement structurés

Contents

CHAPTER 1

Introduction

Does nature make leaps?

A fundamental question lies at the heart of evolutionary biology is the tempo of evolution a slow, gradual crawl or a series of a few abrupt leaps? The debate was sparked by two contemporaneously proposed modes of speciation Darwin (1859) favoured a model of gradualism, wherein speciation results from small changes accumulated over time, whereas Huxley proposed saltationism, where speciation occurs suddenly through substantial phenotypic changes as an explanation for then significant gaps in the fossil record (Lyons 1995) As more and more gaps in the fossil record were filled in, the gradualistic view of speciation increasingly gained acceptance over saltationism Although originally about speciation, the debate between gradualism and saltationism is equally applicable to the process of adaptation It remains to be seen, however, whether gradualism is also a feature of evolution over smaller time scales, such as in adaptation to local environmental conditions Unknown to Darwin and contemporaries, evolution ultimately proceeds through selection on DNA-sequence mutations, which by their nature are discrete However, these mutations can vary in their effect on organismal fitness, with most having a small effect on fitness and only few having a large effect If the fine grained processes of adaptation are observed, such that the effects of individual mutations can be observed, is evolution underlain by a large number of small, similarly-sized changes or by fewer, variably-sized changes in fitness?

Theoretical models of adaptation

First attempts at answering this question were made with theoretical models, which take one of two forms being based either on the evolution of phenotypes or DNA-sequences (Orr 2005a) One of the earliest models of adaptive evolution, a model of phenotypic adaptation, was developed by Fisher (1930), which models organisms as having many traits that must be adapted simultaneously, with an 'optimal' organism having the best possible of each trait This process was captured with a geometric model, where each of the n traits relevant to organismal fitness in a particular environment represents an axis in 'phenotype space', and the intersection point of all axes represents the optimal phenotype (see Figure 1 1) An organism that does not have the optimal phenotype lies some distance s_{opt} away from this optimum, with all points equidistant from the optimum being the same fitness Conceptually, mutations can be treated as vectors in this space that describe both the direction and magnitude of the mutational effect Beneficial mutations are those that bring the phenotype closer to origin, while deleterious mutations move the phenotype farther away Whether a mutation is beneficial or not is not solely determined by mutational direction–the phenotypic effect must also not be so large as to overshoot the optimum state, which would decrease organismal fitness Conversely, mutations that are infinitesimally small cannot overshoot the optimum, leaving only mutational direction to determine selective advantage or disadvantage If the phenotypic effect of a mutation r is mapped to fitness effect through a Gaussian function, the phenotypic effect of a mutation is correlated to its fitness effect Geometrically, the consequence is that 50% of all infinitesimally small mutations are beneficial (Figure 1 1B) This can be readily seen from the expression for the probability p that a mutation of size $r \leq s_{opt}$ is beneficial,

$$p = \frac{1}{2}\left(1 - \frac{r}{s_{\text{opt}}}\right) \tag{1 1}$$

As the model assumes that the wild-type starts out already fairly well adapted (i e s_{opt} is small), the probability that a mutation is beneficial increases as r approaches zero Similarly, the probability of being beneficial also decreases as s_{opt} approaches zero, suggesting that the availability of beneficial mutations decreases as a population approaches a fitness optimum This lead

Fisher to conclude that the bulk of adaptation should be made up of many mutations of small effect

Fisher's model and its interpretation were readily accepted and for decades remained unchallenged, but later work would reveal that although the mathematical reasoning was sound, the conclusion was biologically flawed (Orr 2005a) Fisher missed that a mutation must not only be beneficial in order to contribute to adaptation, it must also survive stochastic loss while at low frequency Termed 'drift', the probability of loss of a beneficial mutation while rare was shown by Haldane (1927) to be proportional to its effect on fitness s $P(\text{loss}) = 1 - 2s$ As a consequence, many mutations of small effect will be lost before they increase in frequency through selection Fisher's oversight was only discovered fifty years later by Kimura (1983), who expanded Fisher's model to incorporate the effects of drift (Orr 2005a)

With Kimura's modifications, the model now predicted that the average effect size of mutations would be larger than Fisher originally thought By corollary, adaptation would involve fewer mutations, however, this reduction is strictly qualitative, because mutations can be infinitesimally small, exact predictions about the number of substitutions cannot be derived from the model Strictly phenotypic models are oversimplified in that they do not take into account that mutations occur in a DNA sequence of fixed finite length, which restricts the total number of possible mutational changes Sequence space models, the second common type of model, capture this process with more biological realism by only permitting mutations that are possible for a given sequence, whether in DNA or amino acid space (Orr 2005a) The most influential of these sequence-space models, the 'mutational landscape model' (MLM), was developed by Gillespie (1983, 1984, 1991) Like in Fisher's model, populations are initially isogenic and start some distance away from a fitness optimum However, instead of modeling the evolution of continuously-valued phenotypic traits, the MLM models transitions between discrete genotypes Adaptation is constrained to a fixed set of genotypes, each with an associated fitness value For a gene or genome of finite length L, there are 4^L possible sequences, which can be assigned a fitness rank i from 1 to 4^L, where smaller i means higher in fitness This can be conceptualized as a walk on an 'adaptive landscape', where the heights of peaks correspond to genotype fitness (see Figure 1 2) A critical assumption of the model is that only $3L$ of these sequences, neighbouring genotypes that can

be reached by a single base-pair substitution, can be transitioned to from the present wild-type at a given time Mutations are assumed to be sufficiently rare to preclude double or triple mutations (i e A to T, C or G is permitted, but not AT to GC) The present wild-type is also assumed to start out well adapted to its environment, so that few of the $3L$ accessible mutations will be beneficial Together, these assumptions put bounds on the number of mutations that are substituted during adaptation, the single-substitution requirement makes it unlikely that the optimal genotype will be reached in a single step, and the assumption that the wild-type is well adapted restricts the maximum number of steps that are beneficial Using simulations, Gillespie concluded that the total length of adaptive walks should be quite short, being between 2 and 5 mutations on average (Gillespie 1991) The condition of high wild-type fitness also allows for assumptions about the distribution of fitness effects of the mutations available to adaptation If the fitnesses of all possible genotypes are arranged into a distribution, the wild-type will be found in the right-hand tail, hence fitter genotypes will be found even farther to the right Draws from the right-hand tail of a distribution are governed by extreme value theory (EVT), a body of probability theory Consequently, Gillespie (1991) showed that the fitness effects of beneficial mutations should be either exponentially (assuming no optimum) or Pareto distributed (assuming a right-truncated distribution of fitness effects, associated with a fitness optimum)

Later work by Orr (2002) has shown that both the effect size of mutations fixed in a single step and the total number of mutations substituted during an entire bout of adaptation should depend weakly on the starting rank of the wild-type (and consequently, its distance from a fitness optimum) If the wild-type rank is i, the expected fitness jump caused by a new mutation is $E[\Delta w] = 2(i-1)\mathrm{E}[\Delta_1]/i$, where Δ_1 is the difference in fitness between the fittest and second-fittest genotypes and depends on the distribution of fitness effects Likewise, an approximate relationship between i the number of substitutions K during an adaptive walk arises from taking the average of so called perfect selection, where the fittest sequence is reached in a single step, and random adaptation, where a random genotype is fixed at each step,

$$K = \frac{1 + \ln(i-1) + \gamma}{2}, \tag{1 2}$$

where $\gamma \approx 0\ 577$ is Euler's constant This suggests that populations starting from lower wild-type fitness should fix both larger and more total mutations over the course of adaptation, compared to populations starting from higher wild-type fitness

Empirical studies of adaptation

Although these models have been in existence for several decades, empirical tests of their predictions have been generally lacking, which in part stems from the lack of a suitable test system Natural populations are not suitable because measuring fitness in the wild is fraught with difficulty and adaptation occurs too slowly to observe in real-time (Orr 2009) Instead, laboratory populations of model organisms have been employed, which, due to their short life spans and simple life-cycles, allow us to study adaptation as it unfolds While probably the first attempt at laboratory studies of adaptation was a study of heat tolerance in monads by William Dallinger (Dallinger 1878, Haas 2000), such studies did not gain popularity until the late 20th century (Buckling *et al* 2009) Contemporary evolution experiments primarily use bacteria, viruses, fungi (including yeasts), or unicellular algae strains to study adaptation to some stress, such as a novel environment or competitor (e g Lenski *et al* 1991, Rokyta *et al* 2008), or to an induced deleterious mutation, such as an antibiotic resistance mutation (e g Schoustra *et al* 2005, 2009) These populations are allowed to adapt to their environment over many generations (usually 500 or more), with aliquots of the population being stored in suspended animation at regular intervals for future analysis Upon the conclusion of the selection experiment, the fitness of populations at each time point can be measured concurrently, and from these changes, the processes of adaptation can be inferred

The majority of these experimental evolution experiments have employed unicellular organisms or viruses as model organisms (Buckling *et al* 2009), but increasingly filamentous fungi are gaining popularity (e g Bruggeman *et al* 2003, Schoustra *et al* 2005, 2006, 2009, Bosmans 2009) The idea of using filamentous fungi as a model system for population genetics is not novel, but traces back to Pontecorvo (1944) who remarked that spontaneously-arising sectors of differing growth rates could be used to study genetic drift and selection One species in particular, the ascomycete *Aspergillus nidulans*,

has been frequently used in studies of adaptive evolution (both as the target organism, Bruggeman *et al* 2003, Schoustra *et al* 2005, 2006, 2009, and as a non-coevolving pathogen of *Drosophila melanogaster*, Wolfle *et al* 2009, Trienens *et al* 2010) *A nidulans* has many of the same properties of unicellular organisms that make it tractable as a study system it grows rapidly, survives storage in suspended animation and is easily cultured An added advantage of using *A nidulans* as a model organism is that it is also capable of sexual and parasexual recombination, allowing beneficial mutations to be easily recombined into different genetic backgrounds Sexual crosses can be used, for example, to estimate the number of accumulated mutations without genetic sequencing (Zeyl 2005) Additionally, because it has only 8 chromosomes and a relatively small genome (30 Mb) compared to other fungi, there is a wealth of knowledge about its genetics, both through classical experiments (Pontecorvo *et al* 1953, Clutterbuck 1969) and modern sequencing techniques (Galagan *et al* 2005)

Mathematical growth models

Despite the recent surge in the use of *A nidulans* as a model organism, there are no published mathematical models describing its growth in a manner useful to evolutionary biology Mathematical population growth models are used to predict the number of individuals in a population as a function of relevant variables (e g time or surface area) and parameters (e g rate of reproduction or resource availability), an example of which is the classic lag-exponential-stationary model of bacterial growth The absence of equivalent models for filamentous fungi can be ascribed in part to the complexity of the fungus life cycle (Nielsen 1992), which unlike most unicellular organisms involves multiple differentiated structures that reproduce at different rates Although modeling fungal biomass instead of absolute numbers of individuals has been one approach taken to avoid these issues, biomass models are inadequate for processes that depend directly on population size, such as mutational supply and competition between genotypes Developing a model based on individuals is critical because it can serve as the foundation for other theoretical models predicting the occurrence and spread of new mutations in fungal colonies and the outcome of competition between different fungal strains in a spatially structured environment

Aims and outline of the thesis

This thesis represents two distinct but not fundamentally disconnected experiments with the fungus *A nidulans* Although the primary motivation behind this thesis was a study of adaptive evolution, further development of *A nidulans* into a model system for studying adaptation requires a better understanding of its growth cycle in a context relevant to population genetics Chapter 2 details an adaptive evolution experiment that studies the effect of distance from a fitness optimum on the properties of adaptive walks These results show that contrary to a gradualist model of adaptation, the length of adaptive walks remains independent of initial distance from a fitness optimum Instead, genotypes farther away from an optimum fix mutations of larger effect

Chapter 3 develops a mathematical growth model for *A nidulans* useful in both fundamental evolutionary biology research and applied science, such as food microbiology and industrial production of antibiotics using fungi Developing growth models analogous to the exponential model of unicellular organisms is required for the continued use of fungi as model organisms for adaptation This model will be used as a basis for further models on the growth and spread of novel beneficial mutations

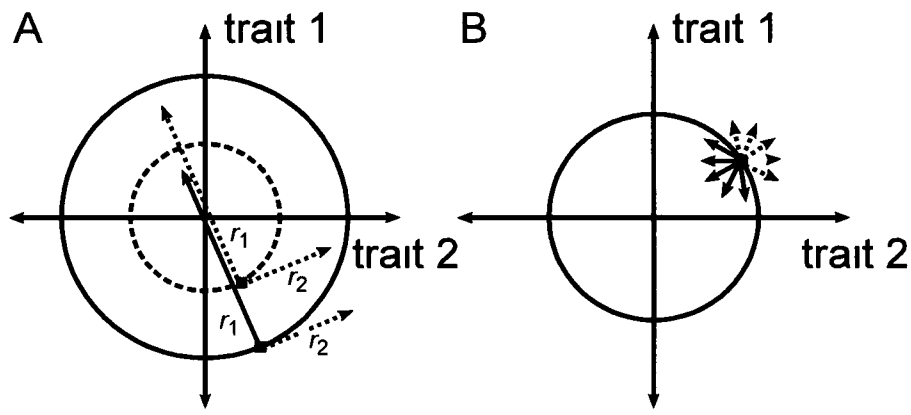

Figure 1 1 Fisher's geometric model can be conceptualized as the movement of a phenotype in n-dimensional space (here, $n = 2$), with each axis corresponding to a trait relevant for fitness The wild-type phenotype is located a distance s_{opt} away from the optimum, represented by the radii of the circles A) Mutations move the phenotype in the space, with beneficial mutations bringing the phenotype closer to the optimum at the origin of the space (solid arrows), and deleterious mutations moving the phenotype away (dashed arrows) Note that mutations with the same magnitude and direction can be conditionally beneficial depending on the wild-type's phenotype B) Infinitesimally small mutations have a 50% probability of being beneficial

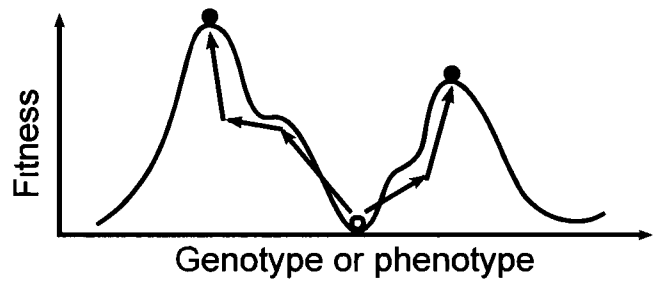

Figure 1 2 Conceptual model of an adaptive landscape Adaptation moves populations across the landscape, a process known as an 'adaptive walk' In contrast to Fisher's model, the process is discrete Height of the peaks corresponds to fitness

CHAPTER 2

Adaptive walks starting different distances from a fitness optimum

Abstract

Adaptation involves the successive substitution of beneficial mutations by selection, a process known as an adaptive walk Little is known, however, about the properties of adaptive walks because beneficial mutations are thought to be rare Gradualist models of adaptation, which assume that all mutations are uniformly small, predict that adaptive walks should be longer when the founding genotype is less well adapted Here we test this prediction experimentally by estimating the length of adaptive walks in evolving populations of fungus that differ in their initial degree of adaptedness We show, contrary to the gradualist view of adaptation, that the length of adaptive walks in all populations is approximately the same, involving just two mutations on average This result arises because poorly adapted populations tend to fix mutations of larger effect, on average, than those fixed by better-adapted populations Notably, we also show that the slope of the relationship between adaptedness and the marginal fitness increases associated with subsequent beneficial mutations fits predictions derived from fitness landscape models of adaptation incorporating the effective number of traits under selection Our results suggest that the length of an adaptive walk may often be independent of the fitness of the founding genotype and, moreover, that poorly adapted populations can quickly adapt to novel environments This

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latter result implies that emerging pathogens, for example, can adapt very quickly because only a few mutations are required, even when fitness is initially very low

Table of symbols

Table 2 1 Symbols used in this chapter

Symbol	Definition
s_{opt}	Distance from fitness optimum, $s_{\text{opt}} = (w_{\text{opt}} - w_m)/w_m$
w_{opt}	Fitness of the fittest genotype, i e the fitness optimum
w_m	Mutant fitness
s_j, s_{j-1}	Selection coefficient of the jth and $(j-1)$th mutation
w_j, w_{j-1}	Fitness of the jth and $(j-1)$th mutants
N_e	Effective population size
m	Number of traits under selection
μ, $(\hat{\mu})$	Resistance mutation rate (estimator)
C	Total cultures plated
z	Number of cultures with a resistant colony
N	Total number of cells

Introduction

The ability to predict the extent and dynamics of adaptation when genotypes of different fitness adapt to the same growth conditions is a crucial task for the theory of adaptation Variation in fitness in a previously well-adapted population can result from an environmental change that causes previously neutral standing genetic variation to have negative fitness effects of varying magnitude How such variation in initial fitness among populations, or among individuals within a single population, affects the process of adaptation to a common environmental challenge remains unclear because it requires knowledge about the number and magnitude of beneficial mutations substituted during adaptation The rarity of beneficial mutations has meant that we know very little about them, and consequently we are still some ways from developing a strong, empirically-validated theory of adaptation

Adaptive evolution can be conceptualized as a series of steps through an adaptive landscape, where height corresponds to fitness, and horizontal position to a genotype or phenotype (Orr 2005a) Adaptation occurs through the subsitution of mutations that increase fitness, moving populations up fitness peaks on the landscape Mutations are subsituted until no further improvement is possible and all new mutations decrease fitness, at which point the population is said to have reached a local fitness optimum The number of beneficial mutations, or steps, required to reach an optimum from different starting points will depend on the magnitude of the fitness increase associated with each step The classic view amongst evolutionary geneticists has been that all mutations are of small effect (Fisher 1930) This so-called gradualist view represents one extreme of a continuum of possible fitness effects associated with beneficial mutations and should lead to walk lengths that increase with distance to the optimum (Figure 2 1A) At the other extreme, walk lengths can be independent of distance to the optimum because mutations are variable in their fitness effect Under this 'variable effects model', large effect mutations will be preferentially substituted when they occur, meaning that they can make up the extra distance to the optimum that would otherwise be covered by many mutations of small effect (Figure 2 1B)

Recent theory using phenotypic and DNA sequence models of adaptation has lent support to this latter idea In the phenotypic approach based on Fisher's geometric model (FGM) (Fisher 1930, Orr 2005a,b, 2006), populations are subject to recurring mutations that effect n traits The optimal combination of traits represents a fitness peak, about which genotypes undergo stabilizing selection, with fitness assumed to fall off as a Gaussian function of the distance to the optimum By contrast, mutational landscape models (MLM) (Gillespie 1983, 1984, 1991, Orr 2002, 2003) treat adaptation as a sequence of moves in DNA space, where transitions from one genotype to another are only possible between single-substitution mutational neighbours Each of L possible DNA sequences is ranked by fitness from $i = 1, 2, \quad , L$ where sequence $i = 1$ is the fitness optimum and less-fit genotypes have higher rank (Orr 2002) Both models predict that the availability of large effect mutations should increase as distance to the optimum increases, suggesting that walk length should be at most weakly dependent on distance to the optimum (Orr 2002) Here we provide what is, to the best of our

knowledge, the first direct test of this prediction

Empirical tests of these interpretations for the effect size of beneficial mutations and their consequences for the length of adaptive walks (*i e* Figure 2 1) require tracking the number and effect size of mutations substituted when adaptation proceeds from different starting fitnesses To accomplish this, we investigated the number and effect size of mutations fixed during adaptive walks by tracking the fitness over time of 100 populations of the filamentous fungus *A nidulans* founded from five genotypes All lineages were selected in a common environment over approximately 1000 generations, by which time they had reached a fitness plateau corresponding to a fitness optimum for their environment The founding genotypes each possessed a single unique deleterious mutation induced by ultraviolet light in a common genetic background Previous work has shown that the unmutated ancestral strain from which the founding genotypes were derived resides on a fitness peak in this environment (Schoustra *et al* 2005, 2006), which means that the difference in fitness between the unmutated ancestor and an evolving population constitutes a measure of distance to a fitness optimum Distance from the optimum can thus be expressed analogously to a selection coefficient of a mutation returning the population to the fitness peak, $s_{\text{opt}} = (w_{\text{opt}} - w_m)/w_m$, where w_{opt} is the fitness of the unmutated ancestor and w_m is the fitness of the genotype with a deleterious mutation Using an experimentally-validated maximum likelihood statistical framework (Schoustra *et al* 2009), we estimated the effect size and total number of mutations fixed by tracking the changes in fitness of each lineage We found that the effect size of mutations scaled with the degree of initial maladaptedness, but that average walk length was not significantly different between different starting genotypes The absolute length of our observed walks across all starting genotypes was quite short, about two steps on average These results constitute strong evidence against the gradualist interpretation of adaptive evolution

Methods

Media and growth conditions

For all experiments we used solid complete medium (CM) adjusted to a pH of 5 8, consisting of $NaNO_3$ 6 0 g/l, KH_2PO_4 1 5 g/l, $MgSO_4$ 7H $_2$O

0 5 g/l, NaCl 0 5 g/l, 0 1 ml of a saturated trace element solution containing $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$, tryptone 10 g/l, yeast extract 5 g/l, agar 10 g/l, and glucose 4 0 g/l (added after autoclaving) Cultures were incubated at 37°C

Strains and fitness mutants

The 'ancestral' strains of *A nidulans*, WG562 and WG145, were kindly provided by Fons Debets and Marijke Slakhorst at Wageningen University, the Netherlands The reduced-fitness strains were generated first through mutagenesis and then selecting progeny resulting from crossing the mutants back with an ancestral strain We generated five reduced-fitness strains by first exposing WG562 (*lys*B5, mycelial growth rate of 13 6 mm/d) to ultraviolet-C light (UVC) Spores suspended in saline-Tween (distilled water with NaCl 0 8% and Tween-80 0 05%) were placed 46 cm away from a UVC bulb (Sankyo Denki G30T8, 30 W, 254 nm) for two minutes, which corresponded to 80% spore killing Irradiated spores were spread on fresh CM and five colonies with reduced mycelial growth rate (MGR) relative to wild-type colonies were sampled We then sexually crossed these five mutant strains with the unmutated strain WG145 (*w*A3, *pyr*A4) and selected five progeny, one from each cross, to initiate the selection experiment The segregation patterns of the cross, coupled with the tendency for UVC to induce C to T point mutations (Pfeifer *et al* 2005), suggested each mutant possessed only a single deleterious mutation The additional step of selecting progeny from the cross helps to ensure that only a single deleterious mutation was present in each starting genotype, had a second, undetected small-effect deleterious mutation been present in the mutant parent, a randomly-chosen progeny resembling the mutant class would have a 1/3 chance of carrying both deleterious mutations, compared with a 2/3 chance of carrying exactly one (assuming the mutations were unlinked) Starting MGR values of the five genotypes, labeled A-E, were 3 42, 4 4, 7 04, 8 18 and 9 mm/d, respectively Adjacent fitness mutants differed significantly from each other, (pair-wise t-tests with Holm-Bonferroni correction, Holm 1979, $df = 23$, $p < 0\ 006$)

Selection experiment and fitness assay

We selected 100 lineages (20 from each of the five fitness mutants) on solid CM Lineages were propagated by serial transfer once every 5 days, or 80 (mitotic) generations, for a total of 12 transfers The initial inoculum was a 5 μl droplet dense spore suspension ($> 10^4$ spores) Population size before transfer reached approximately 10^9 nuclei At each transfer, a 5 μl random sample of each population was inoculated on fresh medium and an aliquot of 0 7 ml mixed with 0 5 ml of 80% glycerol was stored at −80°C Following the selection experiment, the fitness of each lineage was assayed simultaneously in triplicate from frozen samples at generations 80, 160, 240, 320, 480, 640, 800 and 960 (corresponding to transfers 1–4, 6, 8, 10 and 12) by inoculating 5 μl of spore suspension on solid CM

Maximum likelihood estimation of mutations

We used an experimentally-validated maximum likelihood (ML) framework to determine the the number and effect sizes of mutations (see ref 18) The number and effect sizes of fixed mutations are determined from the fitness data by fitting to the data a series of models ($M_1, M_2,$ M_n) with an increasing number of changes in fitness ($1, 2,$ $, n$) The selected model is the model with the largest number of fitness changes n that improves log-likelihood model fit by at least 4 over a model with $n - 1$ changes, which results in a difference in AIC (Akaike 1974) of 6 This approach is conservative with respect to the total number of substitutions, but equally so across the populations derived from all founding genotypes

From the selected models, selection coefficients of each mutation were calculated as the relative difference in fitness, for the jth mutation, $s_j = (w_j - w_{j-1})/w_{j-1}$ (where w_0 is the fitness of the founding genotype) Walk length, the number of mutations fixed over the adaptation experiment, was one less than the number of fitted changes We regressed the number of substituted mutations with founder distance from the optimum For each regression, we include a power analysis, which indicates the probability of rejecting a false null hypothesis given the observed effect size, a fixed rejection criterion ($\alpha = 0\ 05$, by convention) and sample size (n, determined by the experimental method) We also report the minimum sample size n^* that would have been required for power = 0 8, given the same observed effect

size and conventional α

Fluctuation assay

As a check on the mutational supply rate for each starting genotype, mutation rate to acriflavine resistance (concentration of 25 μg/ml of CM, sufficient for preventing growth of sensitive wild-type WG562) was estimated for all starting genotypes using a fluctuation assay (Luria and Delbruck 1943, Jones *et al* 1994, Bosmans 2009) For each starting genotype, approximately 5×10^4 spores were spread on each of fifty plates and the number of plates with growing colonies was counted after 2 days of incubation at 37 °C An estimator for mutation rate μ is $\hat{\mu} = \ln(C/z)/N$, where C is the total number of cultures, z is the number of cultures with no mutants and N is the total number of individual cells (nuclei) in the growing culture of cells before subdivision (Jones *et al* 1994)

Predicted relationship between $s_{\mathbf{opt}}$ and s_j

A quantitative theoretical prediction for the relationship between s_{opt} and s_j exists (Martin and Lenormand 2008, Equation 4)

$$s_j = \frac{4s_{\text{opt}}}{4+m},$$

however a qualitative test of this relationship is not meaningful because both s_j and s_{opt} are constrained to be positively correlated because both depend on the inverse of w_{j-1} Multiplying both quantities by w_{j-1} removes this constraint, instead creating a prediction for the relationship between absolute fitness change and absolute distance to the optimum, which are not constrained to be correlated Since w_{j-1} is not a random variable, we can define $\Delta w_j = \mathrm{E}\left(w_j\right) - w_{j-1}$ and $\Delta w_{\text{opt}} = w_{\text{opt}} - w_{j-1}$, giving the following relationship

$$\Delta w_j = \frac{4\Delta w_{\text{opt}}}{(4+m)}$$

We used linear regression to estimate the empirical slope of the relationship between Δw_j and Δw_{opt} This predicted relationship depends on the number of independent traits under selection, m, which can also be calculated from the reduction of fitness caused by deleterious mutations in muta-

tion accumulation experiments (Tenaillon *et al* 2007) We used data from a mutation accumulation study with *A nidulans* grown under the same conditions (Bruggeman *et al* 2003) to estimate m The calculation of m from mutation accumulation experiments requires that we know the effective population size, N_e Based on bottleneck size and the number of generations between transfers, we estimate a lower bound for N_e of 48, which gives $m = 3\ 76$ A maximum for N_e can be calculated from knowledge of the mean selection coefficient associated with deleterious mutations and assuming that for such mutations to accumulate, $1/s < N_e$ In Bruggeman *et al* (2003), $s = -0\ 0069$, which gives $N_e = 145$ and $m = 11\ 36$ It is notable that these two estimates of m, derived independently, give similar results Thus, for this species in this environment, the number of independent traits under selection appears to be between three and 12 These estimates for m suggest the slope of the relationship of Δw_j and Δw_{opt} should be between 0 27 and 0 57

Results

Fitness trajectories

The fitness trajectories of all 100 lineages are shown in Figure 2 2 Fitness increased though time and the final fitnesses of the lines derived from all starting genotypes did not significantly differ from each other (one-way ANOVA $F_{1,93} = 0\ 62$, $P = 0\ 43$) Three of five starting genotypes had a final fitness that was not significantly different from the fitness of the unmutated ancestor (one-sample t-tests, $P > 0\ 05$) but the remaining two (A and B) were significantly lower ($P < 0\ 0018$) Notably, one lineage from population C (third-fittest founding genotype) reached a substantially higher fitness than all others, including the unmutated ancestor, which is evidence that this lineage adapted toward an alternate adaptive peak, consequently, this lineage was dropped from all analyses, although its inclusion did not change our results qualitatively The variance in fitness among populations across founders also did not differ significantly at the end of the experiment (Levene's test $F_{4,90} = 0\ 13$, $P = 0\ 97$), suggesting that the ruggedness of the underlying adaptive landscape was the same for all starting genotypes

Number of mutations fixed in adaptive walks

The mean number of mutations fixed across all populations was 2 16 ± 0 08 s e m (range 1–4) The relationship between walk length and distance to the optimum was positive but not formally significant (linear regression with error nested within starting genotype slope = 0 13 ± 0 04 s e m, intercept = 2 00 ± 0 07 s e m, R^2 = 0 75, $F_{1,3}$ = 9 10, P = 0 057, power = 0 62, n^* = 7 5, Figure 2 3) Note that the regression is performed by nesting the variation among replicate selection lines (that is, those that share the same ancestor) within starting genotype to prevent over-inflation of residual error, when the data are not nested, the relationship becomes less significant (slope = 0 13 ± 0 09 s e m, intercept = 2 00 ± 0 13 s e m , R^2 = 0 02, $F_{1,98}$ = 2 15, P = 0 15, power = 0 04, n^* = 390) We repeated the above analysis including data on walk length from a separate experiment involving the same wild-type ancestor that carried a costly mutation conferring resistance to a fungicide (Schoustra *et al* 2009) Our inclusion of this data is justified in part because the resistance mutation is a point mutation (Schoustra *et al* 2006), meaning it is in the set of mutations that could have occurred through UV exposure Our results remain unchanged, with an even smaller observed slope than before (slope = 0 08 ± 0 08 s e m, intercept = 2 10±0 14 s e m, R^2 = 0 22, $F_{1,4}$ = 1 1, P = 0 35, power = 0 22, n^* = 33 2)

It is conceivable that we failed to find a significant relationship between walk length and distance to the optimum because we included some strains of extremely low fitness, and so violated one of the assumptions of models of adaptation that rely on the use of extreme value theory To address this possibility we repeated the analysis but restricted attention to only those starting genotypes that we can confidently assume meet the extreme value theory assumption (formally, this means $s_{opt} < 1$, Martin and Lenormand 2008) We found no relationship between walk length and distance to the optimum (linear model with error nested within starting genotype, slope = −0 046 ± 0 29 s e m, intercept = 2 11 ± 0 17 s e m, R^2 = 0 02, $F_{1,1}$ = 0 03, P = 0 9) Thus our results provide little support for the idea that walk length increases with distance to an optimum (*i e* Figure 2 1B and C)

Selection coefficients of fixed mutations

The distributions of selection coefficients associated with each step of an adaptive walk are shown in Figure 2 4 for each of the five starting genotypes For the first step in adaptation, selection coefficients of fixed mutations are largest in the lineages with lowest initial fitness (linear regression on log-log transformed data to meet the homogeneity of variance assumption slope $= 0\ 99 \pm 0\ 12$ s e m, intercept $= -0\ 98 \pm 0\ 09$ s e m, adjusted $R^2 = 0\ 42$, $F_{1,98} = 72\ 3$, $P < 0\ 0001$, Figure 2 4) For all starting genotypes, selection coefficients also decrease in size during subsequent steps Comparing the fit of nested ANOVA models (i e full and reduced) with step number, distance to the optimum, and their interaction as independent variables, the full model including the interaction does not produce a significantly better fit than a reduced model that includes only the main effects of step and distance to the optimum ($F_{1,212} = 1\ 65$, $P = 0\ 2$) By contrast, including both step and distance to the optimum as main effects does improve fit over a model including initial distance to the optimum alone ($F_{1,213} = 54\ 64$, $P < 0\ 0001$), suggesting that effect size is best interpreted as an additive function of distance to the optimum and step number (i e Figure 2 1A)

After a substitution has occurred, distance from the optimum can be recalculated to include the fitness effect of the fixed mutation In this way, we can investigate the effect of distance from an optimum independently of the number of previous mutations fixed Previous research predicts a positive linear relationship between distance to the optimum, s_{opt}, and the selection coefficient of the subsequently fixed mutation, s_f (Martin and Lenormand 2008), however this relationship arises from the calculation of these two quantities, both are inversely dependent on current fitness, w_{t-1} We note however that the absolute change of fitness due to a new mutation [$\Delta w_f = \mathrm{E}(w_t) - w_{t-1}$] is not constrained to be correlated with absolute distance from the optimum ($\Delta w_{\text{opt}} = w_{\text{opt}} - w_{t-1}$) A prediction for the relationship between Δw_f and Δw_{opt} can be obtained from the relationship between s_{opt} and s_f (see Methods) We find a significant positive relationship between Δw_f and Δw_{opt} (linear regression slope $= 0\ 46 \pm 0\ 04$, intercept $= 0\ 01 \pm 0\ 01$ s e m, adjusted $R^2 = 0\ 33$, $F_{1,214} = 108\ 4$, $P < 0\ 0001$, Figure 2 5) A similar pattern holds for the variance among selection coefficients fixed, with the variance in fitness among fixed mutations getting smaller as distance to the optimum decreases Notably, FGM predicts a specific relationship for

this decline in variance $\mathrm{Var}(s_f) = 8ms^2_{\mathrm{opt}}/\left[(m+4)^2(m+6)\right]$, where m is the number of independent traits under selection (Martin and Lenormand 2008) In words, the variance in selection coefficients should increase with the square of distance to the optimum, or equivalently, the absolute value of the residuals should increase linearly with distance from the optimum We observed a relationship between the residuals and distance from the optimum consistent with this prediction (linear regression slope $= 0\ 28 \pm 0\ 02$, intercept $= 0\ 017 \pm 0\ 02$, adjusted $R^2 = 0\ 6$, $F_{1,214} = 316\ 4$, $P < 0\ 0001$ Figure 2 5)

Discussion

Our experiment tracked adaptive walks among replicate lineages founded from five related genotypes of the fungus *A nidulans* evolving in a common environment Previous empirical work has investigated the properties of the first step of adaptation (Rozen *et al* 2002, Sanjuán *et al* 2004, Barrett *et al* 2006, Hegreness *et al* 2006, Kassen and Bataillon 2006, Perfeito *et al* 2007, Rokyta *et al* 2008) and complete adaptive walks (Burch and Chao 1999, Schoustra *et al* 2009, Rokyta *et al* 2009) We extend this work by investigating individual steps of adaptive walks taken by genotypes that vary in their initial fitness in a common environment Our principal findings support the variable effects interpretation of adaptation, under which mutations of larger fitness effect are substituted in genotypes starting farther from an evolutionary optimum This variation in mutational effect allows the average number of mutations fixed during adaptation to be independent from distance to the optimum, so that walk length remains roughly constant across genotypes of different fitness Taken together, these results suggest that a gradualist interpretation of adaptation, where all fixed mutations are of uniformly small effect, is inappropriate as a model of adaptive evolution toward a fitness optimum

Although models of adaptation are often described as resulting from environmental change, we have chosen to study adaptation to a single common environment as a result of the fixation of different deleterious mutations (so-called 'compensatory adaptation') rather than to five separate environments Three reasons justify our approach First, using distinct mutations in the same genetic background allows us to test directly the effect of starting dif-

ferent distances from an optimum without the confounding effects of differences between environments Different environments are likely to differ both in the ruggedness of the underlying adaptive landscape and the number of traits under selection, factors that are difficult (if not impossible) to control experimentally While comparing adaptation in response to a novel environment and compensatory adaptation would be interesting, unfortunately there currently is no way to select environments in such a way to ensure the number of traits under selection and the ruggedness of the landscape would be consistent for all populations Second, a practical advantage of studying compensatory adaptation is that it permits an *a priori* estimate of distance from a fitness optimum, which is not possible when novel environments are chosen Third, direct tests of theory are still possible because models of adaptation assume only that an initial wild-type has experienced a drop in fitness from a previously well-adapted state, they make no formal distinction as to whether this drop in fitness is due to an environmental change or mutation accumulation Indeed, in many senses the MLM description of adaptation is a theory of compensatory evolution, since adaptation is described as the recovery of fitness following a drop in the fitness of rank of the wild type due to an environmental change To the extent that our experimental system mirrors this process, our results are likely to be general

Across all of our starting genotypes, adaptive walks were short, walks were just over two mutations in length on average, even if attention is restricted to the populations that we can confidently assume reached a common fitness peak These results are consistent with other findings of short walks (Lenski *et al* 1991, Zeyl 2005, Schoustra *et al* 2009) however they stand in contrast to other modestly (Paquin and Adams 1983, Rokyta *et al* 2009) and significantly (Wichman *et al* 1999) longer adaptive walks The choice to study compensatory adaptation may explain the observation of short adaptive walks if only a few adaptive changes are required to compensate for disrupting a single gene Although there is a possibility that the optimum may be reached in a single step through reversions, reversions alone cannot account for short walks, only 19 out of 100 lineages fixed exactly one mutation and the probability that all of these were reversions, which occur only once for every 3×10^7 mutations (*A nidulans* has a genome size of approximately 3×10^7) This observation lends further support to the notion that our results are not a unique property of studying compensatory adaptation

In our experiment, we have calculated the distance to the optimum under the assumption that all populations are evolving toward the fitness peak occupied by the unmutated ancestral strain, the implicit assumption being that the local landscape is fairly smooth In a rugged landscape, however, populations starting far from a focal fitness optimum may have a tendency to be located in a valley between alternative adaptive peaks and so selection may drive some replicate populations up neighbouring peaks of different height Although the degree of landscape ruggedness is thought to affect the length of adaptive walks (Orr 2006), variation in ruggedness is unlikely to explain our results for two reasons First, the adaptive landscape appears to be equally rugged for all starting genotypes because the variance in fitness at the end of the experiment across all starting was the same Second, even if we are highly conservative in our assessment of which fitness peak our populations are adapting toward by redoing our analysis using only those lineages with a final fitness within one standard deviation of the mean of all populations (which includes the fitness of the unmutated WG562 ancestor), the relationship between starting distance from the optimum and walk length remains non-significant, with no significant increase in slope (linear regression with error nested within starting genotype slope $= 0\ 15 \pm 0\ 09$ s e m, intercept $= 2\ 05 \pm 0\ 13$ s e m, adjusted $R^2 = 0\ 34$, $F_{1,3} = 3\ 02$, $P = 0\ 18$) Thus, even if the underlying adaptive landscape is more rugged than we have assumed, such that some of the lineages in our experiment have found themselves on alternative fitness peaks, this is unlikely to change our conclusions of no relationship between walk length and distance to an optimum

Our results are also likely to be robust to the duration of our experiment, prematurely terminating the experiment, *i e* before all populations have reached a fitness optimum, should generate a positive relationship between walk length and distance to the optimum instead of no relationship Early on in an adaptive walk, populations of lower fitness fix new beneficial mutations at a greater rate due to increased supply of beneficial mutations (Gillespie 1984, Orr 2006, Schoustra *et al* 2009) that are substituted quickly due to larger average effect size (Bell 2008, Chapter 3) Terminating the experiment before all populations have reached a fitness plateau would thus result in observing disproportionally more fixed mutations in low fitness populations Indeed, this explanation may be an appropriate interpretation of the weakly positive relationship between walk length and distance to the optimum that

we observed in our experiment starting genotypes A and B, which had the highest starting fitness of the five genotypes used, achieved a final fitness that was significantly less than that of the ancestor from which they were derived However, a longer experiment that allowed for more mutations to fix in these high fitness lineages would thus decrease the slope of the walk length-distance to the optimum relationship even more, meaning that our observation of no relationship is thus likely to be conservative

Despite our finding no differences in walk lengths between these five starting genotypes, variation in walk lengths has been previously observed (Paquin and Adams 1983, Lenski *et al* 1991, Wichman *et al* 1999, Zeyl 2005, Rokyta *et al* 2009, Schoustra *et al* 2009) If differences in starting fitness are not responsible, what other mechanisms could drive the number of substitutions made during adaptation? Two candidate mechanisms are evident First, adaptation in a fluctuating environment can increase the number of observed substitutions because the targets of selection are constantly changing, providing more opportunity for adaptation Environmental change can arise either by design (Collins *et al* 2007) or unexpectedly, such as the evolution of cross-feeding on metabolic by-products (Rozen and Lenski 2000) Second, variation in the number of traits under selection could result in adaptive walks of different lengths Having a larger number of traits increases the chance that many traits are maladapted, which in turn increases the number of opportunities for mutations to improve fitness While it would be tempting to assert that more complex organisms should then always take longer walks than more simple ones, the number of traits under selection is a complex interaction between genotype and environment It cannot be assumed, for example, that viruses should always have shorter walks than bacteria or fungi, in fact, some of the longest adaptive walks have been observed in bacteriophage (Wichman *et al* 1999) In principle, this proposition can be tested by varying the number of important traits, either by comparing adaptive walks of one organism across multiple environments of varying complexity, or of organisms of varying complexity to a single environment

It is notable that we observed the fixation of small-effect mutations in all populations, irrespective of their starting fitness This result is somewhat surprising considering that the magnitude of our populations (more than $2\,5 \times 10^5$ nuclei) implies strong clonal interference–the competition among beneficial mutations arising independently in the same population (Park and

Krug 2007, Schoustra *et al* 2009) Clonal interference should bias the mean fitness effect among fixed mutations upward (Rozen *et al* 2002), especially in lineages starting farthest from the optimum where both small and large effect mutations are available Nevertheless, we have observed the signature of clonal interference in the lowest fitness populations, which is suggested by the observation of a modal rather than exponential- or Pareto-like distribution of fitness effects of fixed mutations (Rozen *et al* 2002) (Figure 2 4 C-E) Clonal interference is therefore present in these populations, although it is interesting that it does not seem to be so strong as to prevent all small-effect beneficial mutations from fixing Detecting clonal interference in the lowest fitness populations also suggests that the independence of walk length from distance to the optimum did not result from an inequality of mutational supply between starting genotypes, which can arise if either population size or mutation rates vary wildly between starting genotypes However, these factors are unlikely to be responsible for the finding of no relationship between distance from the optimum and walk length Differences between genotypes in both initial and final population sizes did not differ by more than one order of magnitude We also detected no relationship between starting fitness and mutation rate, with all genotypes having a mutation rate to acriflavine resistance on the order of 10^{-6} to 10^{-7} per individual per generation

Quantitative predictions about the relationship between starting fitness and the number and effect size of mutations substituted are possible and depend on the details of whether adaptive evolution is modelled using an approach based on phenotypes or DNA sequences (Orr 2002, Martin and Lenormand 2008) Although the models guide our thinking about adaptive walks, quantitative tests of both FGM and the MLM would require explicitly knowing key parameters such as the distance to the optimum (s_{opt}), the number of independently evolving traits (m), and the rank in fitness among all genotypes (i), which is not possible for most biological systems, (but *cf* Rokyta *et al* 2008 for bacteriophage) We have tested the qualitative predictions of increased mutational effect size and increased walk length based on these models by using approximations for these parameters Although the results support the FGM prediction of increased mutational size with distance from the optimum, the lack of a relationship between starting fitness and walk length cannot be used to reject the MLM because the theoretical relationship between walk length and i is non-linear, such that the largest

increases in walk length occur when ι is small and level out as ι increases In our experiment, we do not know with confidence what fitness ranks our genotypes represent, except in relative terms, so the model cannot be used to make strong predictions about the relationship between fitness and walk length

Finding no relationship between starting fitness and walk length has important implications for models of host-parasite or pathogen coevolution The results suggest that initially maladapted genotypes can catch up to better-adapted strains within just a few mutational steps, which could help explain the prevalence of antibiotic resistance and the spread of emerging infectious diseases, such as the recent global outbreak of the H1N1/09 strain of influenza However, predictions regarding the size of individual steps of adaptation in unique lineages are unlikely to be consistently accurate because populations starting far from an evolutionary optimum can fix mutations of both small and large fitness effect

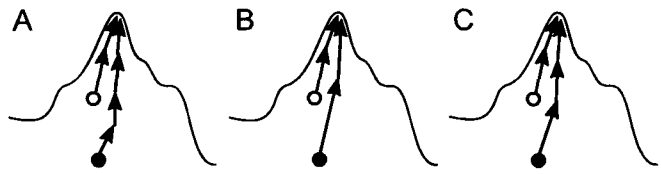

Figure 2 1 Potential effects of different starting fitnesses on adaptation toward a fitness optimum Each arrow represents a single mutation, with effect size proportional to the length of the arrow Genotypes initially more distant from a particular evolutionary optimum (filled point) may fix more mutations (A), larger mutations (B), or both more and larger mutations (C), than a genotype starting closer to a fitness optimum (open point)

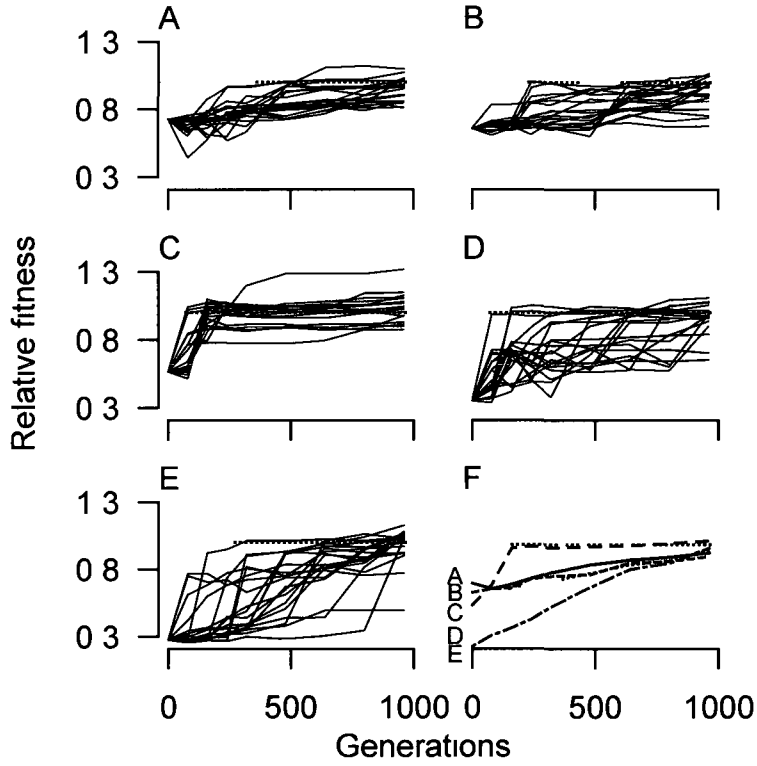

Figure 2 2 Fitness trajectories of evolving lineages, grouped by fitness of starting genotype (A–E) Means of 20 replicate lineages in A–E (F) Fitness is growth rate relative to the growth rate of the unmutated strain WG562 (dotted horizontal lines)

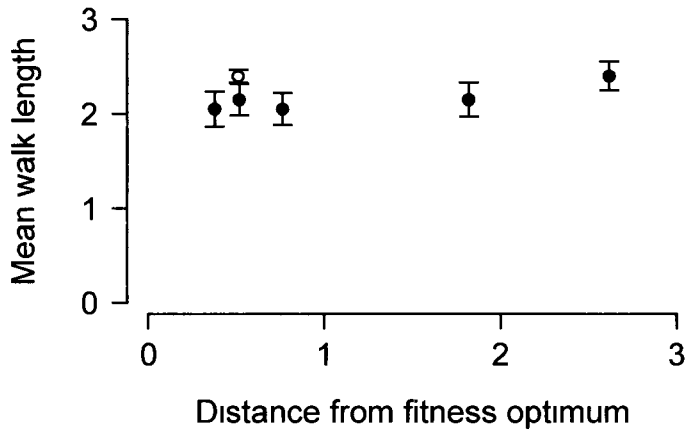

Figure 2 3 Mean walk length (± s e m) of replicate lineages initiated from related genotypes starting different distances from a fitness optimum Filled circles, $n = 20$ replicate lineages, open circle, $n = 58$ replicate lineages from Schoustra *et al* (2009) Points to the right of the dotted vertical line fall outside of the assumptions of extreme value theory

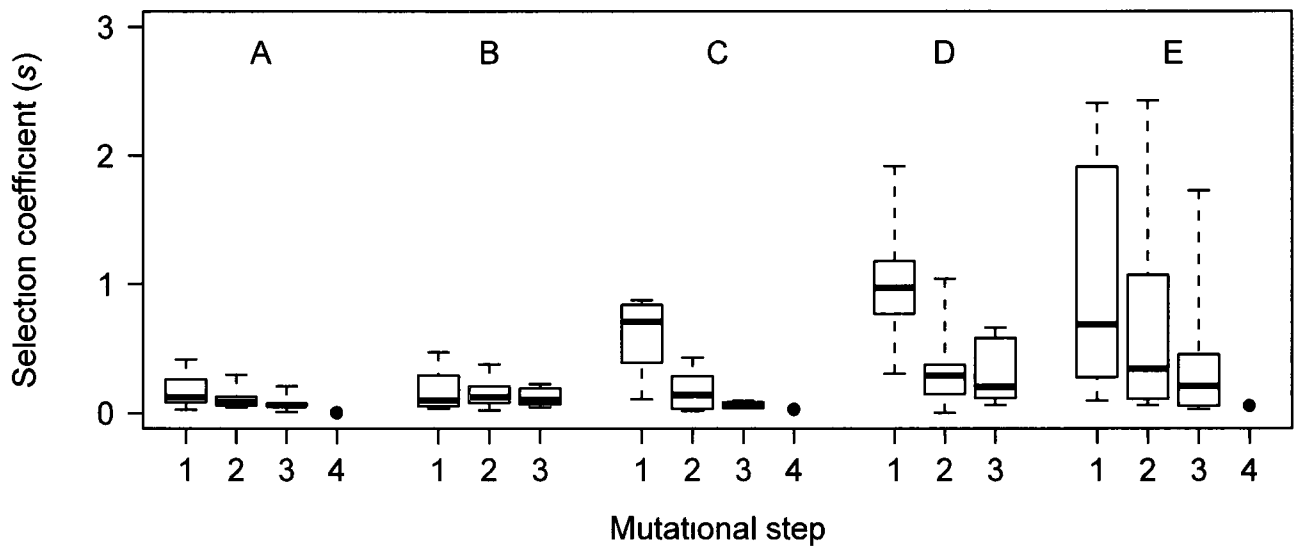

Figure 2 4 Distributions of selection coefficients of mutations fixed in populations evolving from five different but genetically-related genotypes (A–E) Horizontal black bars–medians, box–1st and 3rd quartiles, whiskers–data extremes

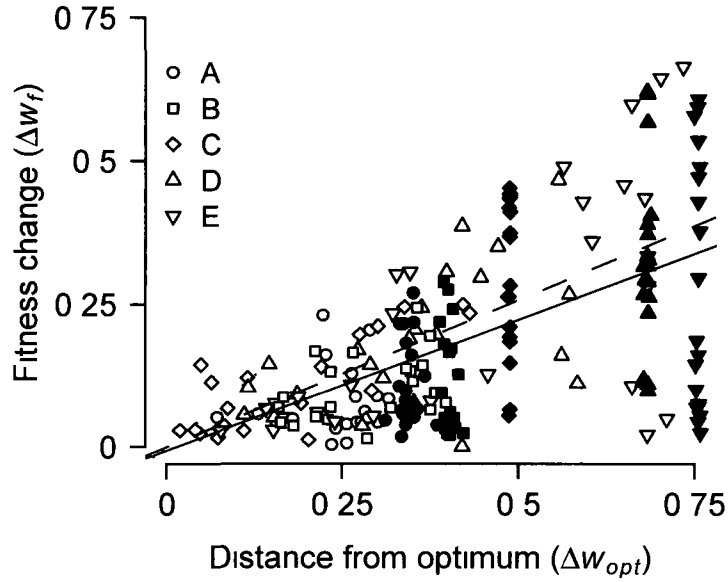

Figure 2 5 Effect size of novel fixed mutations ($\Delta w_f = w_{t+1} - w_t$) increases with distance to the fitness optimum ($\Delta w_{\text{opt}} = w_{\text{opt}} - w_t$) of the genotype in which they arose, for first-step (filled points) and subsequent (open points) mutations Solid line–linear regression (slope = 0 46 ± 0 04, intercept = 0 01±0 01 s e m) Dashed line–predicted relationship $E(\Delta w_f) = 4\Delta w_{\text{opt}}/(4 + m)$ for independently estimated $m = 3\ 76$ (see Methods for derivation) Dotted line–1 1 line

CHAPTER 3

Modelling nuclei number for *Aspergillus nidulans*

Abstract

Filamentous fungi are both common laboratory model organisms and pests of crops and food In many cases, fungi occupy the same functional role as bacteria, but unlike bacteria, we know little about how their population sizes change through time Here we develop a model for the growth of *Aspergillus nidulans* akin to the classic lag-exponential-stationary bacterial growth model We take two approaches to relate changes in the number of nuclei with time in growing colonies Using colonies of different ages, we related the number of colony forming units (CFU), a measure of the number of nuclei, to elapsed growth time and found that CFU is best explained as a squared function of time We then developed a mechanistic growth model for CFU as a function of time based on the physiology of colony growth The full model had three parts, one for each stage of colony development (mycelium only, constant spore production, spore saturation), but a simplification of the third stage of growth also suggested that CFU increases as a squared function of time Although the full mechanistic model did not provide a better fit to the data than the simplified model, it provides insight into when different measures of fitness are most appropriately used

Prepared for submission to *Fungal Genetics and Biology*, coauthored with S E Schoustra

Table of symbols

Table 3 1 Symbols used in this chapter

Variable	
t	time
Parameters	
c	time until spore-producing competency
m	mycelial growth rate
n	nuclei density in mycelium
s	spore production rate
S_{sat}	no of spores per conidiophore at saturation

Introduction

Mathematical population growth models are used to predict the number of individuals in a population as a function of relevant variables, such as time or area, and parameters, such as rate of reproduction or resource availability While several studies have presented population growth models of unicellular organisms (e g Zwietering *et al* 1990, McKellar 1997, Buchanan *et al* 1997), few such models exist for population growth in filamentous fungi Models for population growth have proven very useful in bacteria both in applied and fundamental studies, for example in predicting the number of bacteria over time in food products (Zwietering *et al* 1990) and in modeling the spread of faster-growing mutants arising within bacterial populations (Fitzsimmons *et al* 2010) The development and application of unicellular growth models have been successful because of the simplicity of unicellular life-cycles, which typically involve simple binary fission (Buchanan *et al* 1997) Less progress has been made in the development of growth models for filamentous fungi because their life-cycles are comparatively more complex Although modeling fungal biomass as a proximal measure of population size is one approach taken to avoid dealing with complex life-cycles

(Nielsen 1992), these models are inadequate for processes that depend directly on population size, such as in competition between strains and in mutational supply for adapting populations

In our models, we focus on the increase of the number of nuclei over time as a model for population growth Here we define every nucleus in both mycelium and spores as an individual in the population, since either can disperse to give rise to a new colony Two approaches can be taken to determine an appropriate growth model for filamentous fungi The first approach is to directly measure the number of nuclei capable of forming new colonies (colony forming units, CFU) at progressive time points during colony expansion and then fit statistical models to the data The best-fitting regression model of CFU versus time, corrected for the number of fitted parameters, then describes how population size changes through time This approach is appealing because it directly measures changes in population size, however it is strictly phenomenological and does not provide insight into what physiological changes affect population growth The second approach involves creating a mathematical model for colony growth based on these physiological parameters, which allows for a better understanding of how population size changes through time We employ both of these approaches to develop a population growth model for the filamentous fungus *Aspergillus nidulans* and compare the results of each Under radial growth conditions, the empirical approach suggests that t^2 (i e time x time) is the best model for explaining changes in population size A mechanistic model incorporating mycelial growth rate, the density of spore-producing bodies (conidiophores) and the rate of spore production, suggest that population size is a cubic function of time (*i e* includes t, t^2 and t^3) We find that the mechanistic model provides a good fit to the data but not significantly better than a quadratic model However, the full model simplifies to a quadratic model as colonies age, in agreement with the best-fitting empirical model Finally, we find that colony diameter increases linearly with time, allowing the models of population size to be re-expressed as a function of colony diameter, a good proxy for population size when measuring CFU directly is prohibitive

Experimental procedures

Strain, media and growth conditions

We used *A nidulans* strain WG638 (*yA1*, *ve*A1), a strain well-adapted to growth in the laboratory that produces yellow spores Colonies were grown on solid complete medium (CM) set at pH 5 8, consisting of $NaNO_3$ 6 0 g/l, KH_2PO_4 1 5 g/l, $MgSO_4$ $7H_2O$ 0 5 g/l, NaCl 0 5 g/l, 0 1 ml of a saturated trace element solution containing $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$, tryptone 10 g/l, yeast extract 5 g/l and (added after autoclaving) glucose 4 0 g/l

Estimates of colony diameter and the number of nuclei

Colonies were inoculated as a 5 µl droplet of frozen spore suspension (containing 5×10^5 spores) in the center of a Petri dish Triplicate cultures were incubated at 37 °C for 40, 47, 62, 69, 88, 96, 112, 115, or 120 hours We used the average of two perpendicular measurements of colony diameter as a measure of colony diameter To estimate the number of viable nuclei, we then washed each colony with either 5 or 10 ml (depending on incubation time) of saline tween (0 05% Tween 80, 0 8 g/L NaCl) and thoroughly scraped spores and mycelium from the growth medium with a bent glass rod We diluted the resulting spore and mycelium suspension by 10^{-3} to 10^{-6}, spread 50 µl of each dilution on solid CM with 5% Triton-X100 and counted colonies after approximately 1 5 days of growth at 37 °C

Models

Statistical model fitting

We fit first- and second-order linear regression models with CFU as the dependent variable and elapsed growth time as the independent variable The first-order regression models the increase in CFU with time as a linear relationship, consistent with a constant production of nuclei The second-order regression models CFU production as proportional to area, consistent with a constant density of nuclei across the entire colony surface Akaike's information criterion (AIC, Akaike 1974) was used to compare the fit of these

models, adjusted for additional variables All statistics were performed in R-2 9 2 (R Development Core Team 2010)

Mechanistic model of population growth

Describing the physiology of colony growth verbally is the first step to developing a mathematical model A fungal colony primarily consists of hyphae, asexually produced conidiospores (henceforth, spores) and sexually produced ascospores (Figure 3 1A) A new colony can arise from a single nucleus from any of these three sources, for this reason, we define each nucleus as an individual, although we recognize that other definitions may be more appropriate in different circumstances Undisturbed, the mycelium of a colony growing on a uniform surface expands radially with constant mycelial growth rate (MGR, m), forming a circular colony (Figure 3 1A) Mycelium consists of a branched network of hyphae, which when mature are subdivided by septa into subapical cells, containing around four nuclei each, and apical cells, containing up to 40 nuclei that are mitotically active (Figure 3 1B) Some time after the hyphae have first developed (typically 24 h), some subapical cells differentiate into foot cells that give rise to stalks carrying a spore-head (conidiophore), distributed with uniform density p After a brief period of development, termed 'competency time' (c), specialized cells on the spore-head begin producing asexual spores, each containing one nucleus, by repeated mitoses at a constant rate s A spore head will produce spores until they become saturated (S_m, typically 10,000 to 50,000) at which time spore production ceases In hyphae approximately 5 days old, some subapical cells will initiate a sexual cycle resulting in the formation of a sexual fruiting body (cleistothecium) After around 6 days, the sexual fruiting body is mature and will typically contain 100,000 to 500,000 ascospores In our model, however, we only consider nuclei that are formed during the asexual cycle (i e mycelium and conidiospores) because the sexual cycle takes long to initiate and does not contribute significantly to the total number of nuclei in most experimental designs

Like bacterial growth, which has three distinct stages (lag, exponential and stationary, Edelstein and Segel 1983), this growth process cannot be captured by a single equation Asexual growth can also be broken into three stages mycelium only, constant spore production and spore saturation First,

before conidiophores have become competent, the only contribution to population size comes from mycelial nuclei because no spores are present (Figure 3 2A) Although in reality the highest density of nuclei will be found in apical cells at the boundary of the colony, as a simplification we treat the density of nuclei in the mycelium (n) as uniform, which is justifiable because subapical cells greatly outnumber apical cells For this first part of the model, the number of nuclei is therefore proportional to colony area, which is a function of the square of elapsed growing time Second, after conidiophores become competent, they begin producing single-nuclei spores at a constant rate The time it takes for conidiophores to develop and become competent means that unlike mycelial nuclei, the density of spores varies across the colony surface, since older conidiophores bear more spores This density can be captured as a cone centered at origin of the colony, where the height of the cone is proportional to the number of spores produced over that section of the colony and the base extends across the area of the colony that is competent (Figure 3 2B) Finally, once conidiophores near the centre of the colony have become saturated, spores can be then modeled as a frustum, which is a fraction of a cone with the tip removed (Figure 3 2C) The full model of population size as a function of growing time (t) for these three phases of asexual reproduction is given in Equation 3 1

$$N(t) = \begin{cases} \pi m^2 n t^2 & \text{for } 0 < t < c, \\ \pi m^2 \left[n t^2 + \frac{1}{3} sp(t-c)^3 \right] & \text{for } c \leq t < S_m/s + c, \\ \pi m^2 \left\{ n t^2 + \frac{1}{3} \left[sp(t-c)^3 - (t - c - S_m/s)^2 \left(sp(t-c) - S_m p \right) \right] \right\} & \text{for } t \geq S_m/s + c \end{cases} \tag{3 1}$$

Since the latter parts of Equation 3 1 expand to a cubic function (*i e* contain t, t^2 and t^3 as terms), we also fit a third-order regression model to the data, using the same method as for the empirical models

For older colonies, the final part of Equation 3 1 can be simplified by making two assumptions First, the relative contribution of mycelial nuclei will be low, so they can be ignored Second, as colony diameter increases, the frustum (Figure 3 2C) approaches a cylinder shape, so the 'simplified model' of population size can be described as a cylinder with constant spore density pS_m, $\hat{N}(t) = \pi p S_m [m(t - c - S_m/2s)]^2$, for $t - c >> S_m/2s$ Equivalently, assuming MGR is constant, population size as a function of colony radius (r) can be expressed by replacing t with r/m, $N(r) = \pi p S_m (r - mc - mS_m/2s)^2$

Results and discussion

In this study, we developed a population growth model for *A nidulans*, a filamentous fungus, by using both statistical models fit to measurements of colony population size through time and a mechanistic description of colony growth Whereas previous growth models of filamentous fungi have looked at the increase in fungal biomass (Nielsen 1992, Edelstein and Segel 1983), we focus on the increase in number of nuclei, which is often a more relevant metric of size than biomass (e g for evolutionary biology, invasion of fungi into new habitats, competition between strains, etc) Furthermore, developing a predictive model of the number of nuclei in a colony is necessary because direct measures of population size are only possible by undesirable destructive sampling

A critical assumption of both the statistical model fitting and mechanistic model, we first re-confirmed that mycelial growth rate is constant through time as found by Adams *et al* (1998) Figure 3 3 shows that the relationship between colony diameter and incubation time is linear, consistent with a constant mycelial growth rate (slope $= 0\ 58 \pm 0\ 0024$ S E mm/h, $R^2 = 0\ 9995$, $P < 0\ 0001$) This gives elapsed growth time and colony diameter the same predictive power for population size, and therefore either can be used interchangeably

Our first approach to developing a growth model was through estimating the population sizes of colonies of different ages We found that the relationship between CFU and growing time, was nonlinear (Figure 3 4) and was described best statistically as second-order polynomial of time (Table 3 2) Our second approach was to develop a mathematical population growth model from the growth physiology of an expanding colony, which resulted in the three-part Equation 3 1 Using arbitrary but biologically relevant values for nuclei density, conidiophore density and spore production rate (Adams *et al* 1998), plots of Equation 3 1 qualitatively resembled the empirical data (Figure 3 5) Expanding the latter parts of Equation 3 1 results in a cubic polynomial, which despite resembling the data, does not provide a significantly better fit when the additional parameter t^3 is accounted for (Table 3 2) However, the third part of the model can be simplified to a second-order polynomial for older colonies This cylindrical approximation to nuclei density, which treats nuclei as if they were uniformly dense across

the surface of the colony, underestimates the full model, but the fit improves as colonies age (Figure 3 5) This simplification concurs with the results of the statistical approach

Although the full mechanistic model does not fit the data better, it does provide additional insight into how changes in the physiology of the fungus influence population size Differences in growth physiology can result from changes in the growth environment of a single strain (e g between low and high quality environments, Schoustra *et al* 2010) or can result from mutations altering MGR, spore production or nuclei density, some mutations are even known to have pleiotropic effects on both MGR and spore density (Rosén *et al* 1999) Since population size is directly linked to population fitness, the full model can also be used to indicate which physiological parameters are relevant for fitness under specific circumstances A common approach has been to use MGR as a proxy for fitness (Pringle and Taylor 2002) and the model suggests this is reasonable if mycelial nuclei density, conidiophore density and spore production rate are consistent However, care must be taken in using MGR as a comparison across different environments, which can have disproportionate effects on physiology (e g glucose concentration, which affects spore production but not MGR, personal observation) Certain mutations can also reduce spore density (Clutterbuck 1969), making MGR inadequate as a comparative fitness measure between strains polymorphic at this locus The model does suggest that MGR may best be used as a measure of fitness for closely related strains in a common environment, such as in lineages descending from a common ancestral genotype in an evolution experiment (e g Schoustra *et al* 2005, 2006, 2009), but other components of fitness should be considered when comparing fitness across environments or environmentally-isolated strains with unknown genetic backgrounds

One shortcoming of our model is that it considers only the asexual phase of the growth cycle and does not model the production of ascospores, which are formed as part of the sexual phase We chose to make this simplification because laboratory experiments are often limited to the asexual phase, but the model could be expanded to include ascospore production The production of ascospores is somewhat more complicated to model than conidiospores Asci, each containing 8 ascospores with one nucleus each, are produced in structures known as sexual fruiting bodies or cleistothecia, which begin to develop after 5-7 days of growth (Pontecorvo *et al* 1953) Less is

known about the development of cleistothecia and ascospores than for conidiospores, but expanding the model to incorporate the sexual cycle would require knowing the density of conidiophores (known to vary between genotypes and environments, Adams *et al* 1998, Schoustra *et al* 2010) and the rate of production of ascospores within cleistothecia The model would also have to consider whether mycelial expansion is still occurring at the time of cleistothecium production, because in most experimental setups, cleistothecia only begin to form after the mycelium has already covered all of the available growth substrate (e g in a 90 mm Petri dish, complete coverage would occur within 6-7 days)

Our model also serves as a foundation for developing theoretical models for the occurrence and spread of new mutations in fungal colonies, often seen as mycelial 'sectors' (Pontecorvo 1944) but also occurring in spores (Isaac 1963, Schoustra *et al* 2005) and the competition between different fungal strains in a spatially structured environment Recently (although not a novel idea, Pontecorvo 1944), *A nidulans* and other filamentous fungi are becoming popular model organisms for studies of population genetics and adaptive evolution Such experiments track the evolution of replicate populations, for example, in response to an antibiotic (e g Bosmans 2009) or through the accumulation of mutations that alter organismal fitness (e g Bruggeman *et al* 2003, Schoustra *et al* 2005, 2009) Concrete predictions regarding the fates of individual mutations require a growth model based on individuals, rather than biomass Future work will be the development of a model for the population sizes of these novel sectors as a basis to model their spread in a wild-type population

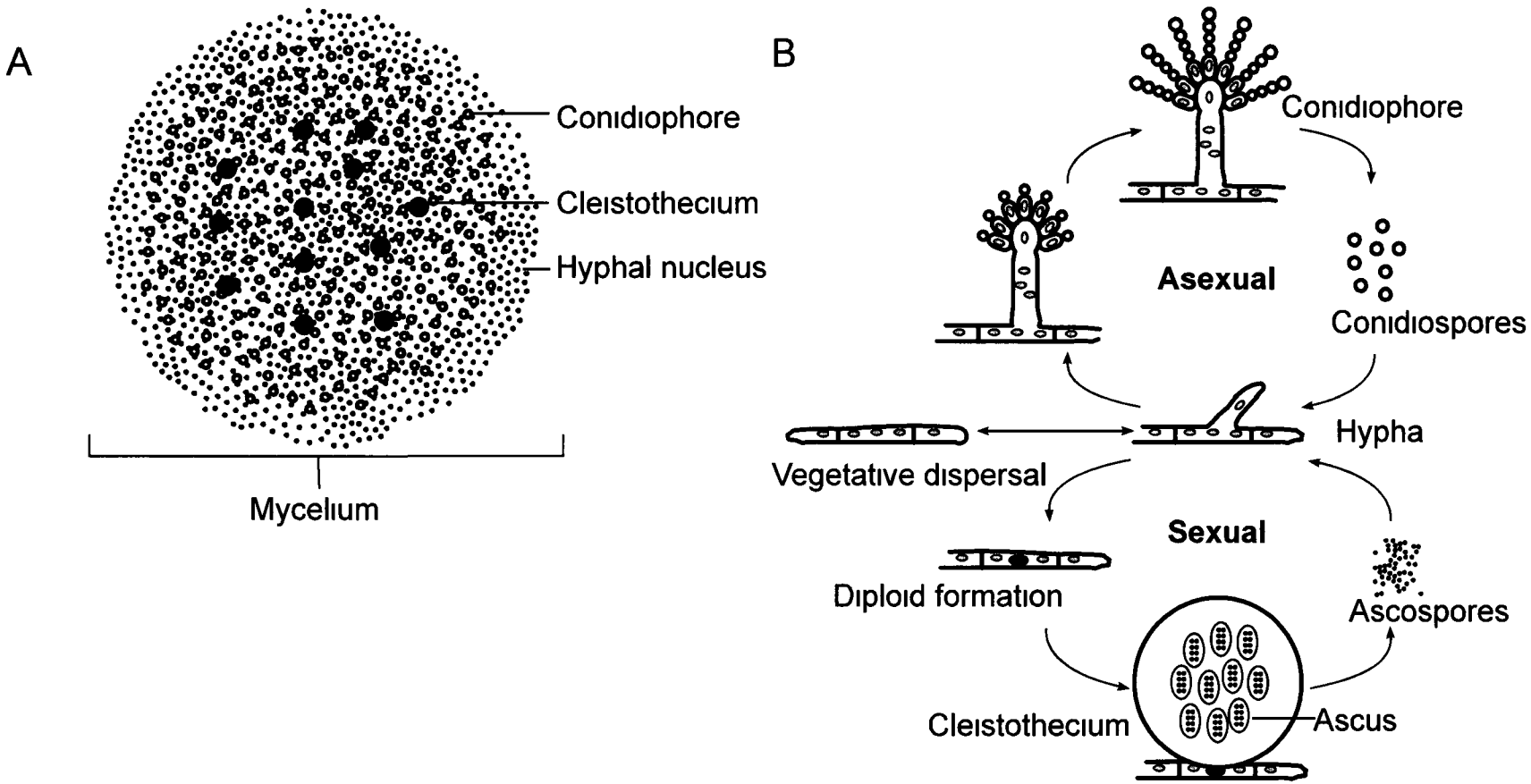

Figure 3 1 Colony structure and lifecycle of *Aspergillus nidulans* A) A colony consists of a vegetatively growing mycelium of hyphae, conidiophores and cleistothecia B) New colonies can arise vegetatively from a mycelial fragment containing a single hyphal nucleus, or through the germination of an asexually- or sexually-derived spore Conidiophores become competent at producing spores about 24 h after they form, and produce spores at a constant rate until they possess 10^4 to 5×10^4 spores A proportion of hyphal nuclei will undergo a sexual cycle, where two nuclei fuse to form a diploid nucleus The diploid nucleus divides mitotically for several rounds before undergoing meiosis to produce haploid ascospores (8 per ascus) Asci are formed within a cleistothesium After around six days, the cleistothecium is mature and contains about 10^5 to 5×10^5 ascospores

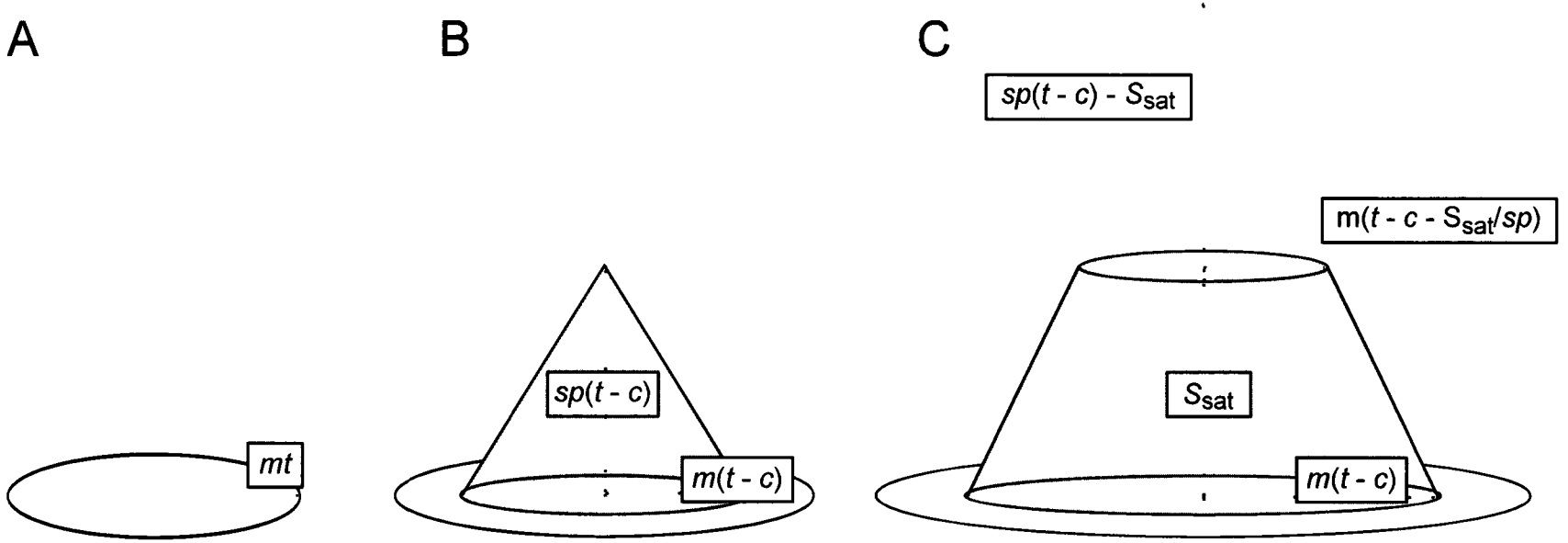

Figure 3 2 Three phases of colony growth, including mycelium (white) and spores (grey) A) Only mycelium, B) Spore production at a constant rate modeled as a cone above the colony, C) Spore saturation modeled as a frustum

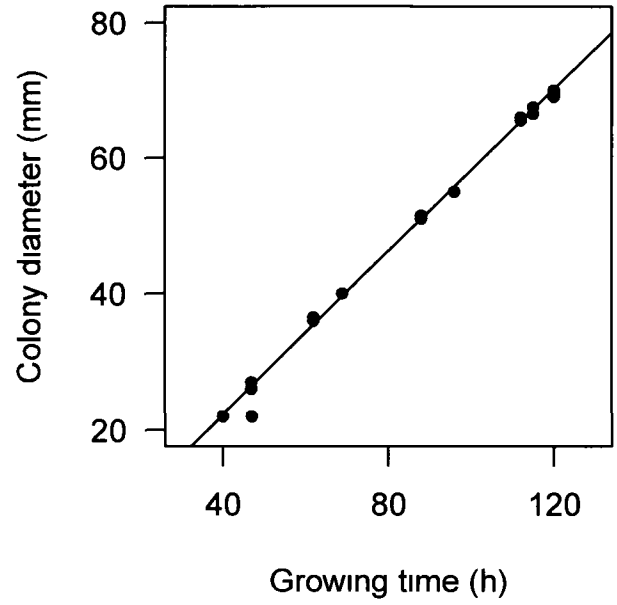

Figure 3 3 Relationship between colony diameter and growing time ($n = 29$, slope $= 0\ 580 \pm 0\ 0024$ SEM, $R^2 = 0\ 9995$, $P < 0\ 0001$)

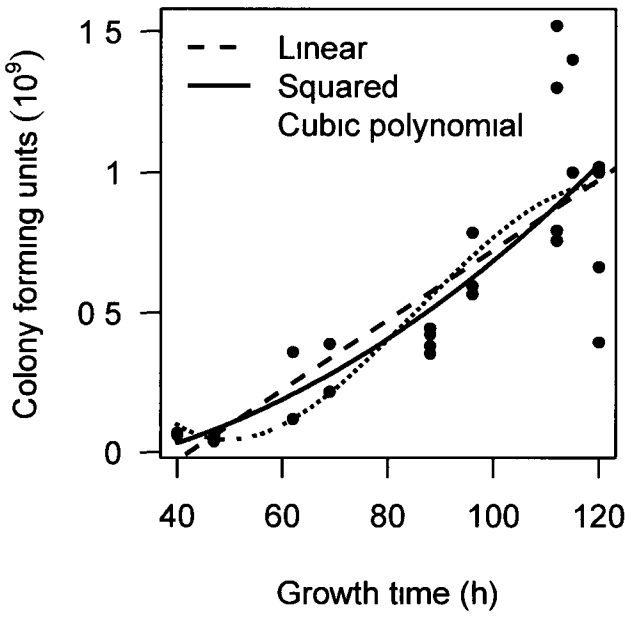

Figure 3 4 Relationship between elapsed growth time and number of colony forming units (CFU), an estimate of the number of nuclei ($n = 29$)

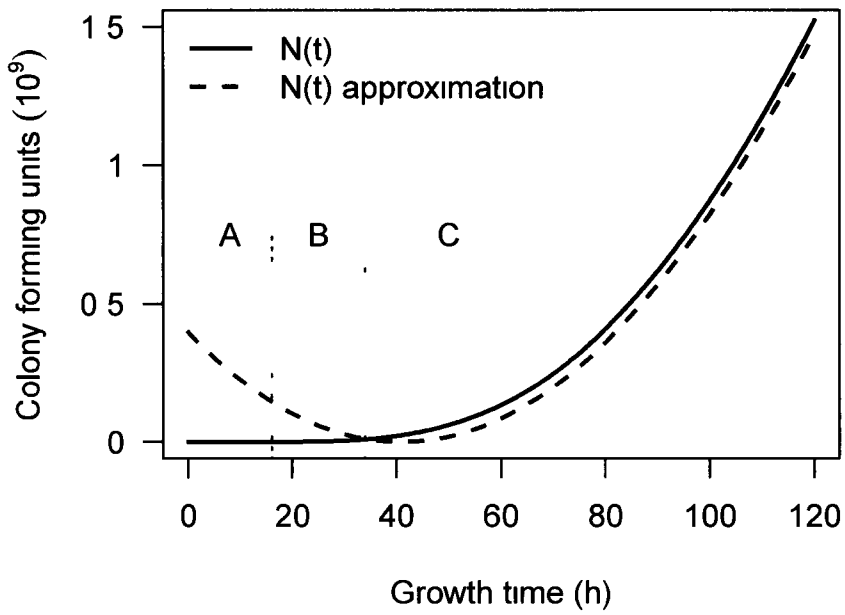

Figure 3 5 Mathematical model based on physiology of colony growth Sections A-C represent the three phases of colony growth (see Equation 3 1) Dashed line is uniform spore density approximation $m = 0\ 5$ mm/h, $n = 300$ nuclei/mm^2, $c = 16$ h, $s = 10$ spores/h, $p = 30$ conidiophores/mm^2, $S_{max} = 500$ spores

Table 3 2 Empirical model fitting of relationship between colony forming units and elapsed growth time

Model	Parameter Estimates	AIC	R^2	F-statistic (df_1, df_2)	Model P
$a_0 + a_1 t^2$	$a_0 = -9\ 11 \times 10^7$ $a_1 = 7\ 77 \times 10^4$ [a]	1216 3	0 718	71 0 (1, 27)	$4\ 9 \times 10^{-9}$
$a_0 + a_1 t + a_2 t^2$	$a_0 = -1\ 84 \times 10^8$ $a_1 = 2\ 61 \times 10^6$ $a_2 = 6\ 17 \times 10^4$	1206 2	0 725	34 3 (2, 26)	$5\ 2 \times 10^{-8}$
$a_0 + a_1 t + a_2 t^2 + a_3 t^3$	$a_0 = 1\ 98 \times 10^9$ $a_1 = -9\ 05 \times 10^7$ $a_2 = 1\ 29 \times 10^6$ $a_3 = -5\ 03 \times 10^3$	1206 4	0 742	23 9 (3, 25)	$1\ 62 \times 10^{-7}$

[a] $P < 0\ 05$

CHAPTER 4

Conclusions

Accomplished through experiments with the fungus *Aspergillus nidulans*, the objectives of this thesis were to illustrate how distance from a fitness optimum influences the adaptive process and to develop a population growth model for *A nidulans* appropriate in the context of adaptive evolution Chapter 2 showed that the total number of mutations substituted during adaptation is insensitive to how initially well-adapted an organism is to its environment Instead of fixing a large number of small effect mutations, adapting populations fix mutations with effect sizes proportional to their distance from a fitness optimum This result is a direct challenge to the gradualist interpretation of adaptation, suggesting that in some cases nature does indeed make leaps Chapter 3, demonstrated that the population growth of *A nidulans* can be predictably modeled by a mechanistic model incorporating its physiological parameters This result is analogous to the classic growth models for unicellular organisms and will allow the number of nuclei present in colonies to be reliably estimated, which has both theoretical and practical applications in evolutionary biology and food science Together, the two experiments also addressed a third, broader goal, to demonstrate the utility and suitability of *A nidulans* as a model system for studying evolution Chapter 2 showed that studies of adaptation are possible with *A nidulans*, and also demonstrated the benefits of working with filamentous fungi, such as the ability to isolate genotypes carrying deleterious mutations and to determine how many mutations they carry through sexual crossing Chapter 3 lays the foundation on which theoretical models predicting how beneficial mutations arise and spread in fungal populations will be built Future work will incorporate areas with different growth rates to predict the spread of beneficial mutations

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Appendix A: Selection coefficients and walk lengths

Selection coefficients of fixed mutations and walk lengths of evolving lineages in Chapter 2

Founder	Lineage	s_1	s_2	s_3	s_4	Walk length
A	1	0 15962	0 29721			2
A	2	0 24752				1
A	3	0 27645	0 05146			2
A	4	0 32589				1
A	5	0 124	0 05416			2
A	6	0 09088	0 08727			2
A	7	0 1193	0 05957	0 20908		3
A	8	0 14908				1
A	9	0 02672	0 12431			2
A	10	0 06812	0 17533			2
A	11	0 08688	0 06052			2
A	12	0 1036	0 28857			2
A	13	0 0626	0 08809	0 07197	0 00558	4
A	14	0 05902	0 06199	0 00904		3
A	15	0 32182				1
A	16	0 08051	0 12758			2
A	17	0 09774	0 12122	0 0609		3
A	18	0 19642	0 04301			2
A	19	0 41456				1
A	20	0 33638	0 06673	0 05685		3
B	1	1 14942	0 17395	0 11374		3
B	2	1 75575	0 0635			2
B	3	0 81518	0 00029	0 66115		3
B	4	1 26865	0 05101			2
B	5	1 01539	0 31735			2
B	6	1 20369	0 20258			2
B	7	0 96073				1
B	8	1 91677				1
B	9	1 90759				1
B	10	0 34181	0 61647			2

Continued on next page

Founder	Lineage	s_1	s_2	s_3	s_4	Walk length
B	11	0 8947	0 31868			2
B	12	0 36369	1 04012			2
B	13	0 97927	0 3813	0 11767		3
B	14	0 81113				1
B	15	0 34713	0 36307	0 5055		3
B	16	0 89671				1
B	17	0 72809	0 53262	0 16992		3
B	18	0 30581	0 26542	0 65652		3
B	19	1 0161	0 12071	0 23343		3
B	20	1 03097	0 28958	0 06116		3
C	1	0 50531	0 07271			2
C	2	0 28615	0 36963			2
C	3	0 87465				1
C	4	0 79487	0 0176			2
C	5	0 35745	0 30186	0 07393	0 02942	4
C	6	0 40725	0 27187	0 03573		3
C	7	0 84435	0 02478			2
C	8	0 12708	0 428			2
C	9	0 10607	0 40863			2
C	10	0 71108	0 13689			2
C	11	0 50612	0 18014			2
C	12	0 54664	0 01562			2
C	13	0 80717	0 12161			2
C	14	0 85738				1
C	15	0 85451	0 03077			2
C	16	0 72408	0 03137	0 04016		3
C	17	0 85082				1
C	18	0 83767	0 15189			2
C	19	0 37404	0 13985	0 09447		3
C	20	0 70975				1
D	1	0 09666	2 42822			2
D	2	0 17407	0 16585	1 72931		3
D	3	0 39548	1 28402	0 10888		3
D	4	2 34338	0 09055	0 03106		3
D	5	2 40771	0 07838			2
D	6	0 2737	0 06602	0 31018		3
D	7	0 27074	0 87169			2
D	8	0 29994	1 32805			2
D	9	1 17267	0 23353	0 4469		3
D	10	1 50739	0 12888	0 07666	0 05853	4
D	11	0 64079	1 03554			2
D	12	2 12159	0 07743			2

Continued on next page

Founder	Lineage	s_1	s_2	s_3	s_4	Walk length
D	13	1 87151				1
D	14	1 95774	0 14785	0 03473		3
D	15	2 243	0 06152			2
D	16	1 70107	0 34351			2
D	17	0 221	2 11188			2
D	18	0 28385	1 03081	0 46599		3
D	19	0 73226	1 10916			2
D	20	0 56677	0 89565			2
E	1	0 09256	0 20399	0 2127		3
E	2	0 45702				1
E	3	0 40523	0 04989			2
E	4	0 08711	0 17683	0 17188		3
E	5	0 08661	0 37787			2
E	6	0 04641	0 31127			2
E	7	0 28955	0 06187	0 04487		3
E	8	0 04164	0 1286	0 1011		3
E	9	0 29608	0 06694	0 0826		3
E	10	0 35713	0 10478			2
E	11	0 27699	0 0914	0 05133		3
E	12	0 08444	0 11801	0 22508		3
E	13	0 47371				1
E	14	0 10269	0 20835			2
E	15	0 05616				1
E	16	0 05337	0 22449			2
E	17	0 21688	0 0202			2
E	18	0 03468	0 15306			2
E	19	0 15578				1
E	20	0 03918	0 10351			2