An Empirical Test of Adaptive Bet Hedging in *Neurospora crassa*

by

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Abstract

Under temporally varying environments, selection is expected to maximize the geometric-mean fitness over generations, which results in the evolution of "bet-hedging" traits such as dormancy. Empirical evidence has been circumstantial and limited to some insects and plants. Here I begin by summarizing the difficulties inherent to the study of bet hedging, and propose the ascomycete, *Neurospora crassa*, as an ideal model system for an empirical test. Cohen's classic 1966 model of the evolution of dormancy is used to ask if selection maximizes the geometric-mean fitness. Populations derived from eleven parental strains were subjected to selection treatments differing in the degree of environmental variance. At the termination of the experiment, I found that dormancy increased with increasing frequency of "bad years," which is consistent with the predictions of Cohen's model. The results provide empirical evidence consistent with the geometric-mean principle, and highlight the need for further work on evolution in variable environments.

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Bet-hedging: Theory and Empirical Tests

1.1 Introduction

Natural environments are variable and known to be stochastic, and environmental variance is expected to drive life-history evolution (Amir and Cohen, 1990; Rebound and Bell, 1997; Tielbörger and Valleriani, 2005; Buckling *et al.,* 2007; Bell and Gonzalez, 2009). Given that fitness is determined by an inherently multiplicative process, natural selection in temporally varying environments will favour genotypes whose traits maximize the geometric-mean fitness (Cooper and Kaplan 1982; Dempster, 1955). This concept is known as the geometric-mean principle, and on theoretical grounds, bet-hedging traits are expected to evolve (Slatkin, 1974; Seger and Brockmann, 1987; Philippi and Seger, 1989). Nonetheless, while many studies inferring the evolution of bet hedging exist, based mostly on anecdotal evidence (for review, see Simons, 2011), direct empirical evidence on bet hedging and the geometric-mean principle is rare. The goal of this thesis is to directly test the geometric-mean principle and thus, bet hedging. The ascomycete, *Neurospora crassa,* a model system in genetics, is proposed here as also an ideal model system for life-history studies. This chapter will begin by outlining why the geometric mean is a more appropriate measure of fitness than the arithmetic mean in variable environments. Furthermore, I explain the importance of studying bet hedging as a likely evolutionary outcome in addition to other responses to fluctuating conditions. Finally, I will argue why *N. crassa* is a suitable model organism for directly testing bet-hedging traits.

1.2 Different Measures of Fitness

Fitness of an organism is determined by how its attributes interact with its environment; the differential persistence of organisms with these attributes is evolution (Burns, 1992). Because natural selection acts on fitness, fitness is a key concept in the study of evolutionary biology (Kingsolver and Huey, 2003). It is imperative then to define fitness accurately and formally, if we are to draw conclusions about evolutionary trajectories and optimality (Burt, 1995; Sober, 2001; Simons, 2002).

Although fitness is well understood as a general concept, there is considerable variation in how it is measured and studied in practice (Stearns, 1976; Denniston, 1978; Cooper, 1984; Reiss, 2007; Roff, 2008). Traditionally, fitness is defined as the extent to which an organism contributes genes to the next generation (Freeman and Herron, 2004), or more technically, it is defined as its average reproductive success across environments (Charlesworth, 1980; Burns, 1992; de Jong, 1994; Sober, 2001). Studies are thus generally based on optimality analysis in which the arithmetic mean fitness, its analog, or a correlated trait is maximized (Orzack and Sober, 1994; Schlichting and Pigliucci, 1996; Benton and Grant, 2000; Orzack and Sober, 2001). Alternatively, fitness measured by the number of offspring per individual of a specific genotype can be used, as it is related to the individual's probability of age specific survival and fecundity and takes into account demographic variability (Nur, 1987; Roff, 2008). However, Roff (2008) points out that these measures are used in models in which the environment is constant, and other situations that include environmental variability may demand a different approach.

1.3 Evolution in Variable Environments

Variable environments will result in changes in optimal trait values or the relative fitnesses of different phenotypes through time (Lande, 1982; Partridge and Harvey, 1988). Addressing the effect of environmental variability in life-history evolution has received considerable attention using a variety of approaches (Ellner and Hairston Jr., 1994; McNamara, 1997;Sober, 2001; Doak *et al.*, 2005). Organisms can respond to environmental variance in different ways. Genetic polymorphism may result from environmental variance, but in contrast to adaptive evolutionary responses to the environment, this phenomenon results from the maintenance of an equilibrium mixture of genotypes, each of which has a high fitness in one environment (Ellner and Sasaki, 1995). Genotypes can exist in such a mixture if there is restricted migration between different environments, or in cases of free migration, if the number of outgoing adults does not depend on the average fitness of the individuals from each environment (Levene, 1953; Seger and Brockmann, 1987). Polymorphism can arise in response to both temporal and spatial variation. The key difference in temporally varying environments is that all individuals will experience all the environments that occur through time (Hedrick, 1995). Given a distribution of genotypes whose fitnesses vary over time, selection will act on life-history traits such that fitness trade-offs will result in individuals whose traits will be selected for under particular changing aspects of the environment. Consequently, genotypes may be specialists with higher reproductive success at particular points in an environmental sequence. Borash *et al.* (1998) showed that early emerging *Drosophila melanogaster* genotypes had higher larval feeding rates but low viability due to density while late emerging types exhibited the reverse pattern.

Similarly, seasonal fluctuations in precipitation resulted in selection for polymorphism in *Linanthus parryae*; Schemske and Beirzychudek (2001) showed that blue coloured flowers had higher fitness in low precipitation years, whereas white coloured flowers had higher fitness in high precipitation years. Although genetic polymorphism may be an outcome of fluctuating selection, it is a population-level phenomenon rather than an adaptive response to unpredictability.

One possible mode of evolutionary response to changing environments is adaptive tracking. Environmental variability may generate strong, fluctuating selection, resulting in frequent maladaptation as average trait values shift away from their optima (Bell and Collins, 2008). However, even if selection is strong on a short time scale, rapid adaptation can still occur provided there is sufficient heritability and standing genetic or mutational variance. This means populations will have genotypes that can survive in novel conditions and thus, adaptively 'track' changes in the environment and can continue to do so as mutation, recombination, or migration replenishes genetic variation (Bell and Collins, 2008). Adaptive tracking may involve the evolution of traits that 'escape' from the effects of environmental change. Franks *et al.* (2006) demonstrated that the annual plant *Brassica rapa* rapidly evolved shorter flowering time in 7 generations in response to multi year drought. Alternatively, spatial heterogeneity interacting with temporal variability can result in the evolution of high dispersal rates; genetic variation is still maintained in the population because rare mutants can invade and specialize in other patches of the environment (Rebound and Bell, 1997; Johst *et al.*, 1999).

Phenotypic plasticity is another response to changing environments, and is defined as an individual's range of phenotypic expression under different environments (Schlichting and Pigliucci, 1998). A function that describes phenotypic changes of a genotype across environmental gradients (Pigliucci, 2001) is known as a norm of reaction. Explanations for how phenotypic plasticity, and by extension, reaction norms arise from fluctuating selection differs slightly. For instance, Via and Lande (1985) using a character state approach demonstrated that plasticity arises due to response to selection for a phenotypic optimum achieved within each different environment. In other words, phenotypic plasticity evolves as a by-product of within-environment adaptation, although limited by genetic correlations of trait values across environments. In contrast, Schlichting and Pigliucci (1998) argue that phenotypic plasticity evolves in response to selection acting directly on reaction norms. Regardless of how plasticity arises, the ability to respond appropriately to environmental cues by altering morphology or phenotype will result in higher average reproductive success across environments (Dudley and Schmidt, 1996).

Adaptive tracking and adaptive plasticity are important responses to environmental variance, but are limited by several factors. The potential for adaptive tracking is dependent on available genetic variation, which is dependent on the supply of mutation and migration of other genotypes. It follows then that evolutionary response to environmental variance requires a large population. If the magnitude of environmental variability is large, the population may decline to the point where reduced genetic variation does note allow for an evolutionary response, which will lead to population extinction (Bell and Collins, 2008). Also, adaptive tracking may be constrained by other

factors. For example, constraints on adaptive evolution such as fitness trade-offs and genetic correlations of life history traits may slow or limit evolutionary response to novel environments (Colautti *et al.*, 2010).

The evolution of adaptive phenotypic plasticity requires predictable environmental change, yet environmental stochasticity is prevalent in natural systems (Boyce *et al.*, 2006; Wilbur and Rudolf, 2006). There have been a number of attempts to incorporate environmental stochasticity into models of plasticity. Plasticity could possibly evolve in response to environmental stochasticity; however, organisms would depend heavily on imperfect information and the reliability of environmental cues would have to withstand disruption from stochasticity (Ergon, 2007). Nonetheless, Turelli and Petry (1980) point out that models taking into account environmental stochasticity assume that such environmental "noise" would not affect arithmetic mean fitness. Furthermore, if the magnitude of environmental variance is too large, especially in stressful environments, the costs of plasticity would constrain its evolution (Van Buskirk and Steiner, 2009). These caveats demonstrate that in the presence of environmental stochasticity, selection may not result in effective tracking or adaptive plasticity.

Under conditions of environmental stochasticity, fitness cannot be maximized across all environments. It is often assumed that, when different traits are favoured over time, traits that maximize the arithmetic-mean fitness, calculated as the sum of n fitness measures divided by n, across environments should evolve. Measuring fitness by the average reproductive success fails to account for the multiplicative nature of reproduction and thus, for long-term persistence (Cooper, 1984; Burns, 1992). In addition, even if the arithmetic mean fitness increases to infinity, the probability of extinction goes to one

(Lewontin and Cohen, 1969; Tuljapurkar and Orzack, 1980). For these reasons, the arithmetic mean is not an appropriate measure of fitness under unpredictable variable environments (Gillepsie, 1977; Frank and Slatkin, 1990; Liou *et al.,* 1993). Another measure of fitness is the geometric mean, which is defined as *nth* root of the product *n* fitness measures and thus, is sensitive to variance in fitness values over generations (Philippi and Seger, 1989). For example, given an organism with non-overlapping generations in a stochastic environment, if the organism were to experience reproductive failure in one particular environment, the arithmetic-mean fitness would still be greater than one while the geometric-mean fitness would be zero, which demonstrates that the arithmetic-mean fitness fails to account for fitness variation as well as the multiplicative nature of fitness in varying environments (Yoshimura *et al.*, 2009; Hunt and Hodgson, 2010). The genotype with the highest geometric-mean fitness will be favoured over the long term (Dempster, 1955). There have been attempts to use the geometric-mean fitness in plasticity models incorporating environmental stochasticity (Behera and Nanjundiah, 1996). However, their stochasticity was "controlled" to include a cyclical, and thus a predictable environment. Behera and Nanjundiah (1996) conclude that beyond a certain point, environmental variability would be too large for phenotypic plasticity to evolve. Traits that increase the geometric mean fitness are suboptimal over short time scales, yet are favoured by selection. Such traits are known as "bet-hedging" traits (Slatkin 1974) and are expected to evolve in response to environmental unpredictability.

1.4 Bet-Hedging Theory

Bet-hedging traits can be classified into two types: conservative and diversification (Philippi and Seger, 1989). A conservative bet hedger expresses a strategy of individual risk avoidance, which is analogous to the adage, 'a bird in the hand is worth two in the bush' (Seger and Brockmann, 1987). This is because a conservative bet hedger avoids phenotypes with extreme fitness values, which reduces variance in mean fitness thereby increasing the geometric-mean fitness. A number of examples of conservative bet hedging traits have been proposed and observed across kingdoms. For example, Einum and Fleming (2004) used simulations from empirical data on egg size in Atlantic salmon (*Salmo salar)* to show that producing larger yet fewer eggs can evolve as conservative bet hedging trait in variable environments. In addition, Simons and Johnston (2003) observed that suboptimal bolting timing in the monocarpic perennial, *Lobelia inflata* may also be a conservative strategy. Similarly, Rees *et al.* (2006) using life history field data of monocarps in the genera *Carlina* and *Caruus* proposed that early flowering evolved to avoid occasional high mortality years.

Diversification, on the other hand, spreads risk among individuals of the same genotype, which would be analogous to 'not putting all your eggs in one basket' (Seger and Brockmann, 1987). The key to this type of bet hedging is that it is accomplished by probabilistic distribution of alternate phenotypes of a trait across all individuals of the same genotype, which allows these individuals to sample a range of different environments through time (Childs *et al.*, 2010). This is what Cooper and Kaplan (1982) termed 'adaptive coin-flipping' because without any cues, a diversified bet hedger will randomly express these alternate phenotypes ('flip a coin') based on the probability of

environments occurring through time. This results in increasing the fitness and phenotypic variances of individuals within a generation, but by averaging over all the fitnesses of the individual phenotypes ('baskets') within each generation, a diversified bet hedger may achieve a higher geometric-mean fitness than a conservative bet hedger (Philippi and Seger, 1989). Examples of traits and studies on diversifying bet hedging are more widespread. Marshall *et al.* (2008) studied the variation in offspring size in broods of marine invertebrates and found evidence of adaptive bet hedging. In insects, multiple mating in females of *Lygaeus kalmii* has been proposed as a bet-hedging strategy to reduce the probability of poor fertilization (Fox and Rauter, 2003). Cáceres and Tessier (2003) discovered variation in dormancy in the eggs of the freshwater cladoceran *Daphnia pulicaria.* There has also been evidence for bet hedging in diapausing insects in which developmental delay can spread the risk of reproductive failure across environments (Seger and Brockmann, 1987; Hopper, 1999). In plants, variation in flowering time as well as in germination time in seeds have been proposed as bet hedging (Clauss and Venable, 2000; Donohue *et al.,* 2005; Simons 2006). In microorganisms, Veening *et al.* (2008) proposed that a trait, exhibiting a switch-like behaviour termed bistability, may be a diversifying strategy. Similarly, bet hedging via phenotypic switching with respect to colony morphology was experimentally observed (Beaumont *et al.,* 2009).

Despite these numerous examples of proposed bet-hedging traits, empirical tests of bet- hedging have been rare (Simons, 2011). Tests of bet hedging require knowledge of the extent of fluctuating selection across generations to ensure that it accounts quantitatively for the response in individual trait variance, and more importantly, it

requires a confirmation that the bet-hedging trait of interest maximizes the geometric-mean fitness under selection (Simons, 2009). With few exceptions, studies have observed trait variance or suboptimality as well as population-level fit between trait variance and fluctuating selection, and thus were only able to infer bet hedging rather than provide direct evidence (Simons, 2009).

Using plant and insect model systems under field conditions to test bet hedging is onerous as these tests would require tracking fluctuating selection as well as fitness effects over generations. The effects of environmental variance can also act on multiple traits, which are often correlated; this makes it difficult to identify the potential bet-hedging traits that evolved (Childs *et al.,* 2010). For example, in the field, flowering time may mediate both a diversifying and conservative bet-hedging response, since flowering size influences reproductive delay, and in like manner, delayed flowering and germination may both occupy the same diversifying bet hedging role, because both reduce variance in recruitment among generations (Childs *et al.,* 2010). Insect studies are similarly problematic. It is difficult to affirm that bet hedging has occurred because fitness costs of some traits can rarely be calculated over a few generations and variation in diapause may actually be attributed to density dependent predation and competition as well as to conditional responses to environment cues as opposed to unpredictable environments (Seger and Brockmann, 1987; Hopper, 1999). Thus far, Clauss and Venable's (2000) work on desert annuals, showing that variation in rainfall accounts for variation in dormancy, Evans *et al.* (2007) work on seed banking, and a study of fluctuating selection on the timing of germination by Simons (2009) provide the most compelling evidence for bet hedging. Dormancy is the most commonly cited bet-hedging

trait in both field and theoretical studies (Evans and Cabin, 1995; Hopper, 1999; Stumpf *et al.,* 2002; Cáceres and Tessier, 2003; Rees *et al.*, 2006; Evans *et al.*, 2007; Malik and Smith, 2008). This is attributable to both the widespread occurrence of dormancy in nature (Sussman and Halvorson, 1966; Clutter, 1978) and to the introduction of a model of dormancy as bet hedging (Cohen, 1966) that has become a classic, and is now known simply as "Cohen's model."

1.5 Cohen's Model

Cohen's model (1966) demonstrates that bet-hedging life-history traits such as seed dormancy is expected to evolve in desert annuals when environmental uncertainty exists. The model is based on a selection regime in which there exist two environments: 'Good' years in which all seeds that germinate survive and reproduce, and 'bad' years in which all germinated seeds die. This model makes a simple prediction: If the occurrence of bad years and good years are random, parent genotypes will evolve a germination fraction that will equal the probability of good years, and a dormancy fraction that will equal the probability of a bad years occurring (Cohen, 1966). When the probability of a good year is greater than 0.5, the average reproductive success for that year is increased by immediate germination, whereas the long-tem geometric-mean fitness is maximized by the evolution of an intermediate dormancy fraction. The simple predictions of Cohen's model—that dormancy should evolve, and in proportion to the frequency of "bad years"—makes this model particularly amenable to empirical test.

For logistical reasons described above, tests of Cohen's model in the field have proven difficult. In addition, results from plant systems in observational studies may be

difficult to interpret because plant life-history traits like germination can be influenced by multiple environmental factors, making it difficult to attribute the evolution of seed dormancy precisely to the effects of temporal variation (Baskin and Baskin, 1998; Childs *et al.*, 2010). Alternatively, the fungal species *Neurospora crassa,* which produces dormant ascospores and has a short generation time, is an ideal model system to ask whether fluctuating selection maximizes the geometric-mean fitness.

1.6 *Neurospsora crassa*: A Model Organism for a Test of Bet Hedging

1.6.1 Placement of *Neurospora* within fungi

The Eumycota (true fungi) traces its origins to the early radiation of eukaryotes more than one billion years ago, and the comparisons of ribosomal RNA suggest they are more related to animals than plants (Wainright *et al.*, 1993; Berbee and Taylor, 1993). Within the Ascomycota, the genus *Neurospora* is grouped under the class of Sordariomycetes, their sac-like ascospore-producing cells known as asci are produced in fruiting bodies known as perithecia (Davis, 2000). It also belongs to the family Sordiaraceae, which is characterized by ostiole (pore) containing fruiting bodies (ascomata) and smooth-walled ascospores with a basal pore and gelatinous sheath. The genus *Neurospora* is distinguished from other genera by its ascospore ornamentation; species of *Neurospora* have ascospores that are broadly fusiform with intercostal veins and longitudinal ribs and that have two germ pores.

1.6.2 History of *Neurospora crassa* as a Model Organism

Shear and Dodge (1927) fully described the sexual stage with their discovery of sexual fruiting bodies (perithecia) and sexual spores (ascospores). By giving a clear account of segregation of mating types at both first and second meiotic division in individual asci, Shear and Dodge were the first to fully characterize *N. crassa* as heterothallic. In addition, Dodge discovered that ascospores would germinate if they were heated at 60°C for 30-60 minutes (Davis, 2000). Later work by Lindegren confirmed Dodge's observations by demonstrating the genetic basis of mating type, confirming second-division segregation, and creating the first linkage maps of *N. crassa* (Davis, 1996). *N. crassa* can be indefinitely maintained in a haploid state, and the famous Nobel Prize winning work done by Beadle and Tatum (1941, 1945) , in establishing the one-gene-one-enzyme model, confirmed that *N. crassa* was a prime model for genetics and biochemistry (Davis, 2000). This resulted in an explosion of research using *N. crassa* such that genetic methods were standardized and discoveries were made on phenomena such as mutation, vegetative incompatability, circadian rhythms and chromosome mechanics (for review see Davis, 1996; Bell-Pedersen *et al.,* 1996; Leslie and Zeller, 1996; Micali and Smith 2003; Salichos and Rotkas, 2010). More recently, the entire genome of *Neurospora crassa* has been sequenced (Galagen *et al.,* 2003).

1.6.3 Biology of *Neurospora crassa*

Due to cumulative experimental work, the biology of *N. crassa*, including its life cycle, is very well known (Figure 1; Davis, 2000). The asexual form is composed of mycelium, a vegetative mass of tubular filaments known as hyphae. These hyphae grow

by extending their tips and contain multiple haploid nuclei that are not separated into distinct cells. As nutrients in the medium are depleted, aerial hyphae bud, branch, and segment to result in macrocondia (asexual spores) that develop a characteristic orange colour due to t cartenoid pigments being produced after exposure to light. Conidia become dry and can be dispersed through air to quickly colonize substrates (Davis, 2000). Well-defined nutrient limitations bring on the sexual cycle (Westergaard and Hirsch, 1954). The sexual stage requires fusion between strains of opposite mating types, A, and a, and takes about 22 to 27 days to complete (Bell-Pederson, 2006). The mating type of *N. crassa* is determined genetically by the mating locus *mat*, which modulates sexual nonself recognition, postfertilization development, and nuclear identity (Raju, 1992; Perkins *et al.,* 2001). Fertilization is preceded by one parent becoming the 'female' by forming a protoperithecium. Filaments called trichogynes will protrude from the protoperithecium that fuse with a hypha or a conidium of the opposite mating type. Trichogynes are attracted by pheromones released by the cells of the opposite mating type, a phenomenon known to be regulated by the mating-type locus (Staben, 1996). Following fusion, the nuclei of opposite mating types pair and undergo simultaneous divisions prior to nuclear fusion and the formation of a brief diploid stage of the *N. crassa* life cycle. The diploid nucleus undergoes meiosis within the ascus, followed by a mitotic division to yield eight nuclei. A second mitotic division occurs and nuclear pairs are packaged into eight ascospores. Ascospores are known to survive for periods greater than 10 years due in part to protective spore layers that are not found in conidia (Sussman and Halvorson, 1966).

Since *N. crassa* exists largely in a haploid state, identifying and using mutants for studies of other biological aspects, including measuring recombination, may be done with relative ease (Davis, 1996; Davis, 2000). *N. crassa* is a superior model organism for tests of Cohen's model because haploid genotypes are more directly exposed to selection parameters. In fact, *N. crassa* exhibits differential growth responses to variation in heat (Lingappa and Sussman, 1959). For example, the circadian rhythm in *N. crassa* can be maintained by compensating for temperature changes while maximizing the rhythm of conidiation output at 25°C, yet temperature changes can result in phase shifts, decreased temperature compensation, and diminished or disrupted amplitudes of conidiation output (Liu *et al.,* 1997; Ruoff and Rensing, 2004). Similarly, heat shock proteins (HSP) regulated by heat shock transcription factors (HSF) and trehalose, a storage sugar in conidia and mycelia, play a role in developing thermotolerance in response to heat (for a complete review see Tereshina, 2005). The mycelia of *N. crassa* grow optimally at about 39°C but will produce HSPs maximally at 45°C. This allows its vegetative structures to survive at otherwise lethal temperatures of 50°C (Plesofsky and Brambl, 1985).

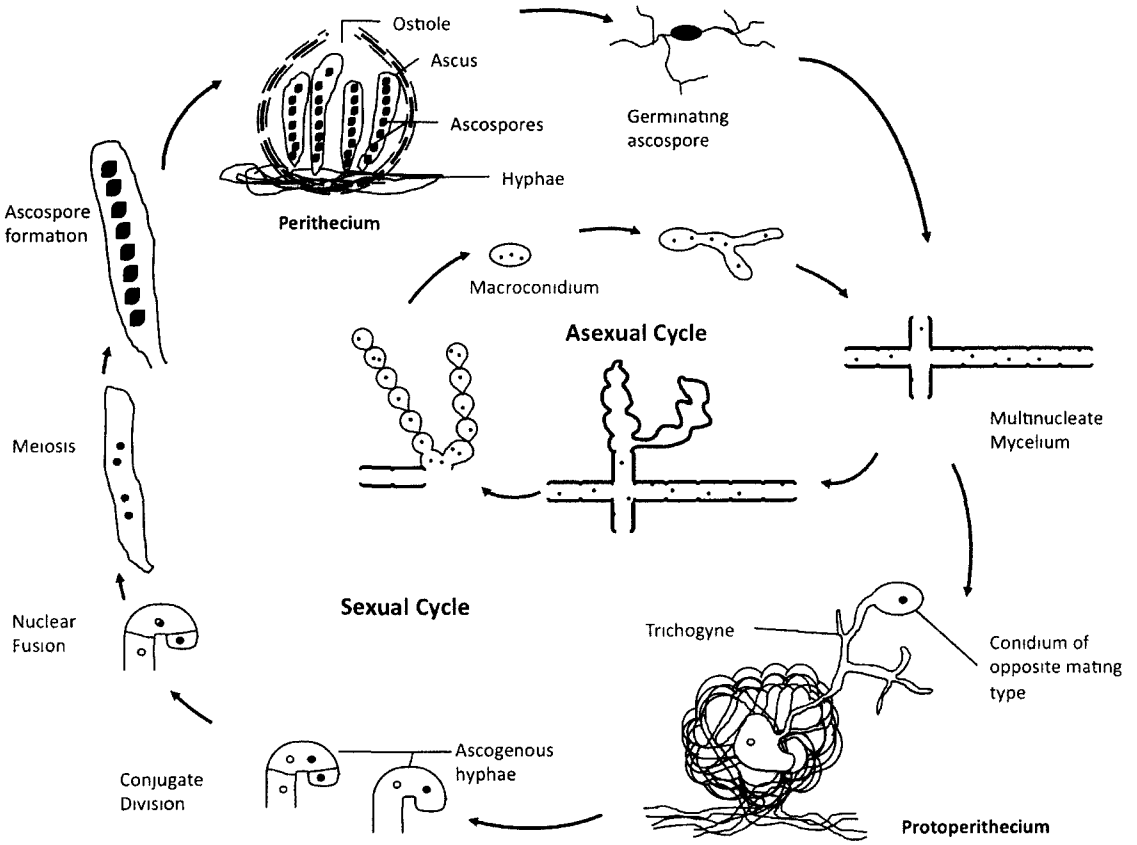

Figure 1. Drawing of the life cycle of *Neurospora crassa* showing both sexual and asexual cycles. Adapted from Davis (2000).

However, the heat sensing network of *N. crassa* is complex, requiring interaction of other proteins with HSPs and HSFs (Sequina *et al.,* 2010). Thus, any genetic variation in the underlying genes responsible for HSPs and HSFs and their regulators can not only alter the heat requirements for post-germination growth and conidial development but also affect the survival of those vegetative structures in differing heating conditions (Thompson *et al.,* 2008). In addition to genetic variation, conidia and mycelia are still relatively sensitive to temperature changes, such that moderate heat stresses during mycelial growth and conidial germination combined with low carbon stress may result in cell death (Plesofsky-Vig *et al.,* 2008).

In contrast, heat shock proteins are absent in heat activation of ascospores (Davis, 2000). Nonetheless, unlike vegetative cells, ascopores contain high levels of trehalose, which acts as a storage 'dormancy sugar' and protects the enzyme trehalase from inactivation at high temperatures; this suggests that ascospores exhibit higher thermotolerance than conidia and possible variable responses to heat activation (Sussman and Halvorson, 1966; Hecker and Sussman, 1973; Tereshina, 2005). It is known that heat-activating spores in the laboratory at 60°C for 30-60 minutes can result in near 100 % germination of dormant ascospores (Bell-Pedersen, 2006). Heat activation brings about key physiological changes such as trehalase and other enzymes metabolizing endogenous substrates like trehalose for energy. This results in substantially increased respiration and protein synthesis and the rigid structure of spore walls degrading to allow the emergence of the germ tubes (Sussman and Halvorson, 1966; Hecker and Sussman, 1973). Spores begin to germinate 3 to 5 hours following this heat treatment, and it is at this early stage of germination and ensuing vegetative growth that exposure again to high temperatures

can be lethal (Lingappa and Sussman, 1959; Bell-Pederson, 2006). Given that kinetics of germination can vary (Sussman and Halvorson, 1966), it follows that ascospore populations can vary in their optimal germination fraction, dormancy, and their temperature profile or norm of reaction. This has been explored experimentally in a close relative of *N. crassa, N. tetrasperma*, which showed that ascospores have particular temperature thresholds and that suboptimal heat conditions would result in germination fractions of below 100% (Lindgerden, 1932; Goddard, 1935). This behaviour may also be reflected in the natural life history of *Neurospora*. Dormant ascospores of a congeneric species, *N. intermedia*, are adapted to being activated by heat following a forest fire (Pandit and Maheshwari, 1994). A global study of *N. crassa* populations has revealed that this species has adapted to a number of different sub-tropical and tropical regions (Turner *et al.*, 2001). Furthermore, Jacobson *et al.* (2006) discovered that *N. crassa* can also exist in temperate climates in both Europe and North America and noted that strains were genetically distinct from region to region.

The biology of *N. crassa* thus makes it suitable for testing Cohen's model and adaptive bet hedging. Heating conditions can be modified to ensure that dormancy in spores, as opposed to heat resistance, is selected specifically, since germinated ascospores, mycelia, and conidia can be killed while dormant spores not activated under a previous heat shock are activated. Furthermore, the selection parameters can be easily controlled in a laboratory due to the extensive knowledge and ease of use of *N. crassa* as a model system. The goal of this thesis is to use *N. crassa* to ask whether dormancy evolves in proportion to environmental stochasticity as predicted by Cohen's model, and to thus provide a direct test of bet hedging and the geometric-mean fitness principle.

Chapter II

Empirical Evidence For Adaptive Bet Hedging in *Neurospora crassa*: A test of the Geometric-Mean Principle

2.1 Introduction

The increasing recognition that stochasticity is a fundamental property of natural environments that cannot be ignored in evolutionary models (e.g. Halley, 1996; Gonzalez and Descamps-Julien, 2004) has been accompanied by a greater focus on how organisms respond, both directly and evolutionarily, to this environmental variability (Doebeli et al., 1997; Petchey *et al.*, 2002). Two well-known forms of evolutionary response are adaptive phenotypic plasticity (Lochmiller *et al.*, 2000; Pigliucci, 2001) and adaptive tracking (Bell and Collins, 2008). The evolution of plasticity and adaptive tracking under unpredictable or rapid change is limited by the availability of standing genetic variance and by the reliability of environmental cues (Barton and Turelli, 1991; Behera and Nanjundiah, 1996; Ergon, 2007; Simons, 2011). Genetic polymorphism is a population-level response to fluctuating selection and, although it can result from variable selection, it cannot be considered an adaptive response (Seger and Brockmann, 1987; Hopper, 1999). Under circumstances that limit the evolution of tracking and plasticity such as environmental unpredictability, the evolution of "bet-hedging" traits is expected (Philippi and Seger, 1989; Lewontin and Cohen, 1969; Frank and Slatkin, 1990; Simons, 2002).

The concept of bet hedging, as well as its evolutionary relevance, is often misunderstood. As a measure of fitness, the arithmetic mean does not take into account the multiplicative nature of reproductive success in the long term as well as variance in fitness values under fluctuating selection through time (for further details, see Chapter 1), but an appropriate measure of fitness is given by the *nth* root of the product of *n* fitness values over generations, or the geometric-mean fitness (Dempster, 1955; Gillepsie, 1977;

Yoshimura and Jansen, 1996; Simons 2002). Characters that maximize the geometric-mean fitness rather than the arithmetic-mean fitness are known as 'bet-hedging' traits (Slatkin, 1974). Bet-hedging traits are suboptimal under average conditions, but because they reduce the variance in fitness over generations by spreading or avoiding risk, they have the effect of maximizing the geometric mean fitness (Seger and Brockmann, 1987). Bet hedging theory is thus central to evolution under environmental change and to the concept of optimality (Simons, 2002).

Bet hedging can be accomplished in two ways: via risk spreading through phenotypic diversification on the individual genotypic level, or via the expression of a "safe" or conservative strategy (Seger and Brockmann, 1987; Philippi and Seger, 1989). Examples of bet-hedging traits include diapause (Philippi and Seger, 1989; Menu *et al.*, 2000), multiple mating in parents (Fox and Rauter, 2003), variance in offspring size (Einum and Fleming, 2004; Marshall *et al.,* 2008), timing of egg laying (Caceres and Tessier, 2003; Einum and Fleming, 2004; Crean and Marshall, 2009), flowering or germination time in seeds (Clauss and Venable, 2000; Simons. 2006), seed banking (Evans at al., 2007) and 'colony switching' or 'bistability' in microorganisms (Veening *et al.*, 2009; Beaumont *et al.,* 2009).

Although numerous bet-hedging traits have been proposed, few tests of bet-hedging theory have been performed (Hopper, 1999; Simons, 2009). This is because a full test of bet hedging is difficult, and would require assessment of the extent of fluctuating selection across generations, and whether the bet-hedging trait is optimal (i.e. maximizes geometric-mean fitness) in a quantitative sense under the amount of fluctuating selection observed (Simons, 2011 submitted). Instead, most tests infer bet

hedging by observing the existence of suboptimality in traits or by showing either qualitative or quantitative agreement between fluctuating selection and trait variance at the population level (Philippi, 1993; Simons and Johnston, 2003). Although these studies provide some evidence for bet hedging, that is the existence of high trait variance, or that the degree of population-level trait variance corresponds to the extent of fluctuating selection, they do not provide direct evidence of bet hedging such that environmental variability accounts quantitatively for individual level trait variance (Simons, 2009). Tests of bet hedging using plant and insect model systems under field conditions are difficult because they require evaluating both environmental variance and its fitness effects over multiple generations (for a summary see Chapter 1). Nonetheless, the most complete evidence for bet hedging so far comes from monocarpic annual and perennial plants (Claus and Venable, 2000; Evans *et al.*, 2007; Simons, 2009; Childs *et al.*, 2010). Dormancy defined as any rest period or reversible interruption of the phenotypic development of an organism, is widespread in nature (Sussman and Halvorson, 1966), and became the most commonly cited diversification bet-hedging trait following the publication of Cohen's (1966) classic model.

Cohen's (1966) model provides an ideal foundation upon which an empirical test of the geometric-mean principle may be performed using a short-lived model organism. Cohen (1966) models a simple case, in which there exist two types of years: 'Good' years in which seeds that germinate produce a new generation of seeds, and 'bad' years in which germinated seeds die without successfully reproducing (Cohen, 1966). Letting Y represent the average yield of seeds produced per germinated seedling in a good year, G the germination fraction, D the constant decay rate, S the number of seeds present, and P_Y

the probability of Y occurring (a good year), over N years the long-term growth expectation, an analogue to the geometric mean fitness, is expressed as (Cohen 1966):

$$\lim_{N\to\infty} \frac{\log S_N}{N} = P_Y \log[G \cdot Y] + (1 - P_Y)\log[(1 - D)(1 - G)] \quad (1)$$

By maximizing the long-term growth expectation with respect to the germination fraction G, it can be shown that G is equal to P_Y (Cohen, 1966). Thus, by the geometric-mean fitness principle, the fraction of seeds that germinate each year is expected to be equal to the probability of a good year and, conversely, the fraction of seeds that remain dormant will equal the probability of a bad year. Because, in contrast, the short-term expected growth rate is always maximized by immediate germination (i.e. zero dormancy) when the probability of a good year takes any value greater than 0.5, Cohen's model is ideally suited to distinguish between the maximization of the geometric-mean fitness from the maximization of the arithmetic-mean fitness. A simple yet fundamental experimental test of Cohen's (1966) model, and thus of bet hedging, would be to ask first whether dormancy evolves when a bad year is expected (i.e. $0 \leq P_Y \leq 0.5$) second, whether there is an increasing trend in evolved dormancy fraction with the proportion of bad years and finally, for a quantitative test (Orzack and Sober, 1994; Orzack and Sober, 2001; Simons, 2009) of bet hedging, whether evolved dormancy fraction is equal to the probability of bad years.

Despite the simplicity of Cohen's model, a conclusive test based on field observations would be difficult. For example, evolved dormancy fractions are difficult to

determine because germination fractions may be influenced directly by environmental conditions, and seeds that do not germinate are not necessarily dormant. The physiology and development of plant seeds are complex such that the interactions of multiple factors influence dormancy and germination (Baskin and Baskin, 1998). Germination is influenced not only by environmental variation through time, but also by spatial variation in soil composition, and nutrient and water conditions (Baskin and Baskin, 1998). One of the corollaries to Cohen's model is that seeds that do not germinate under good conditions are expected to germinate in later years (Philippi and Seger, 1989) under identical conditions. Even if dormant seeds were tracked, years will not be identical and cannot be defined as either good or bad in nature. Finally, natural selection acts on multiple correlated life-history characters, and separating the effects of fluctuating selection from other effects on dormancy would be challenging. A study using a model species with a short generation time under controlled conditions has the potential to overcome many of the difficulties of observational field studies. Thus, an experimental selection approach to test Cohen's model, although it cannot address the issue of how common bet hedging is in nature, provides a unique opportunity to ask whether fluctuating selection maximizes the geometric-mean fitness.

A selection approach using microorganisms that exhibit spore dormancy is an alternative to observational study of angiosperm systems for tests of bet hedging, since the work on the physiology and biology of spores make them useful for investigations into dormancy (Sussman and Halvorson, 1966). The fungal ascomycete, *Neurospora crassa*, in particular serves as an ideal model experimental system for several reasons. First, *N. crassa* produces dormant ascospores (Davis, 2000). Second, dormancy can vary

in response to environmental variance such as changing heat conditions, which is also reflected by the existence and survival of *N. crassa* in multiple climates (Lindegren, 1932; Goddard, 1935). Third, the biology of *N. crassa* is well understood, and it is a model system amenable to well defined controlled experiments (Davis, 1996; Davis, 2000). Previous work with the bacterium *Pseudomonas fluroescens* has shown that phenotype switching, a potential bet-hedging trait, can arise de novo in response to selection in experimental populations (Beaumont *et al.*, 2009). However, direct selection for a trait assumed to be bet hedging does not provide evidence that the trait maximizes geometric-mean fitness or that it is a bet-hedging trait. A test of bet hedging requires establishing a microcosm in which populations are subjected to environmental unpredictability and asking whether bet hedging evolves because it is favoured under fluctuating selection and whether traits that maximize geometric-mean fitness prove to be favoured over generations without biased sampling of individuals that exhibit the assumed bet-hedging trait of interest.

Here, I ask whether fluctuating selection favours the arithmetic- or geometric-mean fitness in a test of Cohen's (1966) model using ascospore dormancy in *N. crassa*. I allow dormancy fraction to evolve independently in each of twelve genetic crosses, each subjected to five selection treatments that differ in the frequency of "bad" years. At the termination of the selection experiment, I compare final dormancy fractions among selection treatments to test the prediction that evolved dormancy fractions are positively related to the frequency of bad years. A positive result would provide evidence consistent with the geometric mean principle.

2.2 Materials and Methods

2.2.1 Overview

The goal of this experiment is to test empirically Cohen's (1966) model for adaptive bet hedging using *N. crassa.* Eleven wild-collected strains were selected that originated from various geographical origins and of opposite mating types (Turner *et al.*, 2001; McCluskey, 2003). With the selected strains, several different crosses were possible, each involving strains from differing geographical origins, and presumably, encompassing different genetic backgrounds. Pairs of wild-collected strains were mated to obtain ascospores and establish selection lines for the selection experiment. Temperature and duration of heat treatments were determined to identify conditions that result in intermediate dormancy of ascospores. It is on this variation in dormancy that fluctuating selection, imposed by different sequences of "good" and "bad" years, may act in a test of Cohen's (1966) model.

2.2.2 Stock Preparation

Eleven wild-collected strains were cultured on Vogel's (1964) minimal medium containing 1.5% sucrose and 1.5% agar; 7 were of mating type A and 6 were of mating type 'a' (Table 1). A conidial suspension was created for each of the strains, and an aliquot of 10 microlitres of conidial suspension was used to inoculate pairs of strains on 1x Westergaard and Mitchell's (1947) synthetic crossing medium (SCM) with 1.5% sucrose and 1.5% agar. In total, 12 crosses were performed using defined strain pairs (Table 2) and each of these crosses gave rise to perithecia that would eject ascospores onto the walls of the crossing tubes.

Table 2.1 Summary of wild-collected strains for the selection experiment.

Strain (MLS)[1]	Mating Type	Geographic Origin
4828 (M89)	a	Asikro, Ivory Coast
4832 (M93)	a	Eremankono, Ivory Coast
4831 (M92)	a	Grabiokok, Ivory Coast
4711 (M65)	A	Haut Diquini, Haiti
4834 (M95)	A	Issia, Ivory Coast
4712 (M66)	a	Leogane, Haiti
P4493 (L36)	a	Lousiana
P4481 (L81)	a	Lousiana
2225 (M31)	A	Mauriceville, Texas
4835 (M96)	A	N'Douci, Ivory Coast
4714 (M68)	A	Pescaili, Haiti
4837 (M97)	A	Sakota, Ivory Coast
4827 (M88)	A	Tiassale, Ivory Coast

[1]Strains referenced from Fungal Genetics Stock Center, University of Kansas Medical center. Alternate labels in parentheses from the laboratory of Myron Smith, Carleton University.

Table 2.2 List of pair-wise crosses used to establish selection lines.

Mating Type *a* Strain (Origin)	Mating Type *A* Strain (Origin)
M93 (Eremankono, Ivory Coast)	M88 (Tiassale, Ivory Coast)
M66 (Leogane, Haiti)	M96 (N'Douci, Ivory Coast)
M66 (Leogane, Haiti)	M68 (Pescaili, Haiti)
L36 (Lousiana)	M97 (Sakota, Ivory Coast)
L36 (Lousiana)	M65 (Haut Diquini, Haiti)
M66 (Leogane, Haiti)	M31 (Mauriceville, Texas)
M89 (Asikro, Ivory Coast)	M68 (Pescaili, Haiti)
M89 (Asikro, Ivory Coast)	M88 (Tiassale, Ivory Coast)
M92 (Grabiokok, Ivory Coast)	M95 (Issia, Ivory Coast)
M92 (Grabiokok, Ivory Coast)	M97 (Sakota, Ivory Coast)
L81 (Lousiana)	M97 (Sakota, Ivory Coast)
M66 (Leogane, Haiti)	M65 (Haut Diquini, Haiti)

An additional cross was done using ascospores bulked together from the other 12 crosses. This cross is referred to as the "mass mating" cross. For this, about 100-150 ascospores were used from each of the 12 crosses to ensure an equal contribution to the mass mating cross. The combined ascospores were heat activated at 60°C for 60 min and then transferred to 1x Westergaard and Mitchell's (1947) crossing medium. This heat activation regime was used to ensure equal contribution from all ascospores in establishing the mass mating cross, since it gives close to 100 percent germination (Bell-Pedersen, 2006; and pers. obs.). In all then, thirteen different genetic crosses were thus available for use in the selection experiment.

2.2.3 Experimental Parameters and Protocol

Three major parameters had to be established for this selection experiment: Heat treatment conditions that result in intermediate dormancy, conditions defining 'good' and 'bad' years, and the environmental sequences to be used in the experiment.

Ascospore Activation Conditions

The first challenge was to find an appropriate heat treatment temperature and time of exposure combination that results in intermediate dormancy fractions. A heat treatment of 60°C for 60 minutes yields maximum germination, but it is known that alterations of the duration and/or temperature can result in intermediate dormancy fractions (Lindegren, 1935). Experiments on varying heat treatments have been performed in a related species, *N. tetrasperma* where it was found that at 60 minutes, temperatures beyond 50°C are needed to overcome dormancy in ascospores (Goddard, 1935; Lindegren, 1935). In addition, a single heat treatment had to fulfill three

requirements. First, heat treatment must kill all hyphae and conidia, thus ending the previous "year." Second, previously dormant ascospores had to be activated. Third, heat activation of newly produced ascospores had to result in intermediate dormancy to mark the beginning of a new year. A heat treatment of 55°C for 45 minutes was found to be effective in meeting these requirements.

For each heat treatment, an aliquot of ascospores was placed in a tube containing 2ml of 1x Vogel's medium containing 0.4% agar, 0.025% glucose and 0.025% fructose. After the initial heat treatment at 55°C, 45 minutes, the tubes were vortexed, and immediately half of the volume was transferred to 1x Vogel's agar medium containing 0.025% glucose, 0.025% fructose, and 1.5% sorbose in a petri dish for analysis of germination and dormancy frequency. The remaining ascospores in the tube were allowed to germinate and were incubated at room temperature (21°C to 24°C) for 48 hours before exposure to a second heat treatment at 55°, 45 minutes. The tubes were vortexed at high speed for 2.5 minutes and then, plated in petri dishes on 1x Vogel's (1964) at 1.5% agar, 0.025% glucose, 0.025% fructose, and 1.5% sorbose for colony counting. Dormant ascospores are defined as those that germinate following the second heat treatment. Dormancy fraction is thus defined as the count of dormant ascospores over the total viable ascospores available, which is the total number of ascospores germinating over the two heat treatments (Table 2.3). One cross, M93xM88, exhibited a high error in mean dormancy fraction due to the low number of replicates sampled compared to other crosses (Table 2.3).

Table 2.3. Mean dormancy fraction of each cross as well as the mean dormancy calculated over all of the crosses.

Cross	Mean Dormancy Fraction	Error (±)
M93xM88	0.3247	0.1772
M66xM96	0.3198	0.0701
M66xM68	0.4243	0.05352
L36xM97	0.3628	0.08347
L36xM65	0.2959	0.1041
M66xM31	0.3516	0.07732
M89xM68	0.2793	0.04134
M89xM88	0.5021	0.05430
M92xM95	0.2329	0.08657
M92xM97	0.2318	0.07032
L81xM97	0.2660	0.08937
M65xM66	0.1050	0.03712
Mass Mating	0.4404	0.05372
Overall	0.3142	0.02331

To avoid the transfer of vegetative material from one "year" to the next, it was particularly important that the experimental heat treatment consistently kills 100% of hyphae and conidia. To test this, conidia and mycelial fragments from a culture of a randomly selected cross (M66xM96) were suspended and 400 µl aliquots were placed in sets of tubes. These tubes were immersed in a water bath at a temperature of 55°C with the exception of three tubes that represented no heat treatment. In the first trial, tubes were removed from the bath at five-minute intervals for up to 60 minutes and plated in petri dishes with 1x Vogel's (1964) at 1.5% sucrose and 1.5% sorbose for subsequent colony counts. In the second trial, the tubes were removed at 2.5-minute intervals and plated. The suspensions for which there was no heat treatment were diluted 10 000x prior to plating. From this experiment, it can be seen that with the exception of one instance at 50 minutes, vegetative material does not survive after 40 minutes of exposure to 55°C (Figure 2). A heat treatment of 55°C for 45 minutes thus satisfies all requirements for the selection experiment.

Defining "Good" and "Bad" Years

For consistency with Cohen's (1966) model, we hereafter use the terms, "good year" and "bad year" to refer to two contrasting environmental states that favour germination and favour dormancy, respectively. The conditions for 'good' and 'bad' years must be defined such that good years allow sufficient time for a complete ascospore-to-ascospore life cycle following heat activation, and such that bad years allow ascospore germination but are brief enough to prevent the production of new ascospores. The average ascospore-to-ascospore generation time for *N. crassa* ranges between 22 to

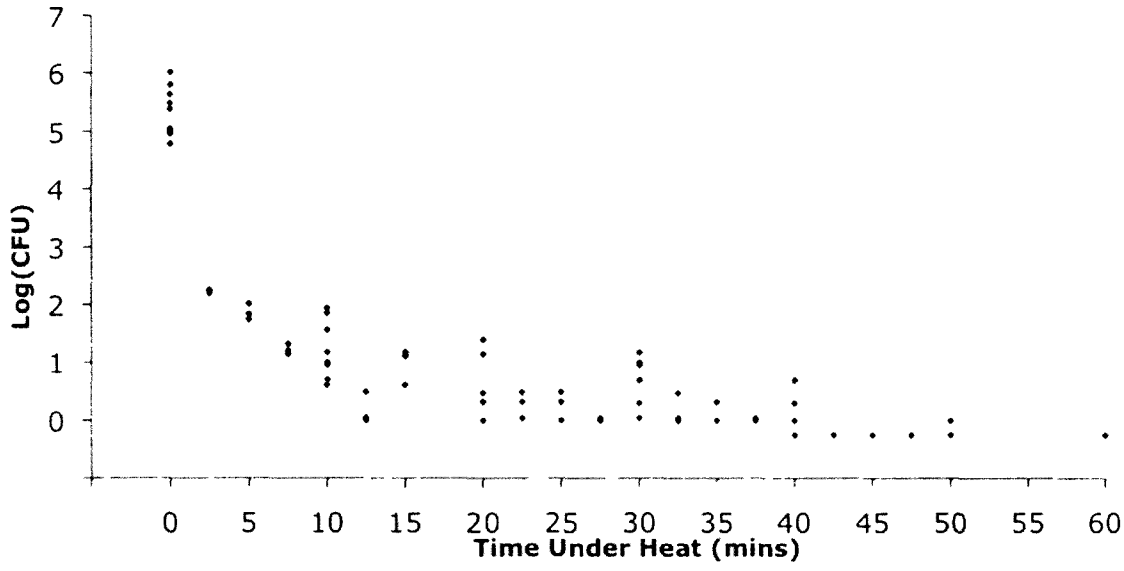

Figure 2. Number of conidia and mycelial colony forming units that survived different durations of heat treatment at 55°C. There were two trials: One done in 5-minute intervals and the other in 2.5-minute intervals. Beyond 40 minutes, no colony units formed with exception of one at 50 minutes.

27 days (Bell-Pedersen, 2006). Each perithecium produces, on average, 200-400 ascospores at 25 °C (Raju and Griffiths, 2008).

To find a suitable duration for a good year, three replicates each of four crosses were made and incubated at 25°C. Following inoculation, petri dish lids were replaced on days 19, 22, 26, 38, and 46. This provides a cumulative total number of ascospores ejected onto the lid for the intervening periods. With the exception of the cross, M96xM66, the largest number of ascospores ejected was during the 19-22 day interval (Figure 3). From this, 22 days was selected as the minimum duration for a 'good' year. As confirmed by preliminary assays (above), hyphae and conidia are highly sensitive to the elevated temperatures required to initiate ascospore germination (Bell-Pedersen, 2006). In fact, following heat activation, ascospore germination takes place within 3 to 5 hours (Goddard, 1935). A 'bad' year was thus imposed by a second heat activation at 48 to 60 hours following the first heat treatment. To maintain similar conditions over the course of the selection experiment, the ascospores, regardless of year type, were germinated in test tubes containing 1.5 ml of 1x Westergaard and Mitchell's (1947) liquid SCM with 1.5% sucrose.

Environmental Sequences And Harvesting Procedure

Cohen's model (1966) predicts that dormancy is selected for in direct proportion to the probability of unfavourable or 'bad' years. Thus, five selection line treatments differing in the frequency of 'bad years' were established (Table 2.4): 0% for 10 years, 20% for 12 years, 30% for 14 years, 40% for 14 years, and 50% for 14 years. The overall mean dormancy prior to selection was close to 30% (Table 2.3). To ensure that response to selection could be attributed to frequency of year type and not to specific year-type

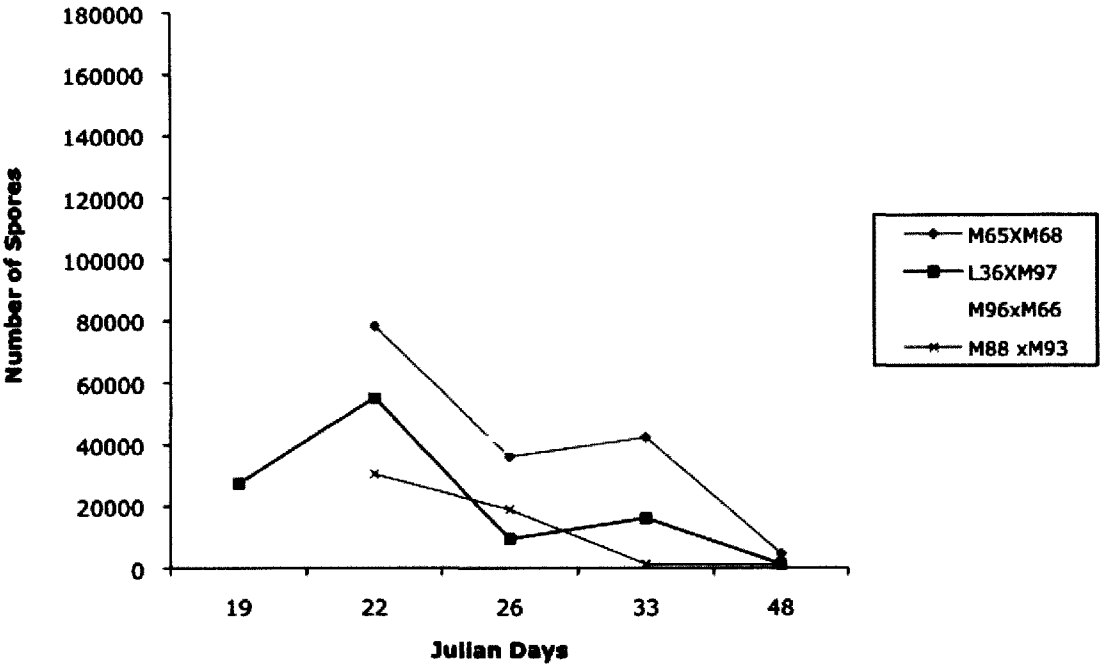

Figure 3. The number of ascospores ejected onto petri dish lid during 5 time intervals. At each time point, each lid was removed and each day point represents the cumulative ascospores ejected during the time interval between lid replacements. With the exception of the cross, M96xM66, there is a relative decline of ascospore production after day 33.

sequences, five randomized year sequences served as replicates within the 20, 30 and 40% selection treatments. Year-type sequences were constrained by the necessity to avoid the occurrence of two consecutive bad years; thus, all sequences for the 50% treatment were the same, alternating between good and bad years.

At the end of each year, ascospores were transferred into a fresh tube containing 1.5 ml of 1x Westergaard and Mitchell's (1947) SCM with 1.5% sucrose. To maximize ascospore capture, tube contents were serially washed through sterile fusible fleece filters. Ascospores were allowed to settle for 30 minutes, and all but 400 µl of the supernatant was then decanted. This effectively reduces the number of conidia in the ascospore suspension. A 100 µl aliquot of ascospore suspension was transferred to a new test tube, and the remaining suspension was stored at 4°C to act as a 'fossil record' for future study of the time course of evolutionary change. All ascospores were harvested after the final year for storage at 4°C.

Five selection line treatments (representing 0, 20, 30, 40, and 50% bad years) were thus each represented by five randomized sequences where possible. Each randomized sequence was replicated using thirteen different genetic crosses, for a total of 325 independently evolving populations. A major advantage of the experimental design is that response to fluctuating selection on dormancy falls out automatically when year t+1 is inoculated using proportional representation of surviving ascospores from year t. This is because relative fitness associated with germination and dormancy is given by the number of ascospores of both types at the end of a year. Out of the 325 samples, 136 samples were contaminated and 101 became extinct. Thus, 88 samples survived for analysis

2.2.4 Post Selection Assays and Data Analysis

To compare germination and dormancy fractions of all selection lines before and after selection, tubes were randomly assigned into five different groups to be heated. Following the same protocol as the initial assays (see Ascospore Activation Conditions), the selected lines were tested for germination and dormancy fractions. Original crosses were tested alongside the selected lines for each group. A one-way mixed effects ANCOVA was used to test if dormancy fractions increased positively with increasing bad year frequency and to test if the effect of bad year frequency was dependent on type of cross used selection line treatment. In addition, to take into account dormancy of the crosses prior to selection, net change in dormancy for each selected line was calculated by subtracting the initial mean dormancy of each cross prior to selection ('before') from each dormancy fraction of the same cross 'after' selection line treatment regime. Thus, to test to see if bad year frequency is a positive predictor of the net change in dormancy, a one-way mixed effects ANCOVA was performed. All statistical tests were performed using JMP (SAS Institute 2003). To confirm some of the differences among treatments in response to selection, a one-way ANOVA was performed using a Tukey's HSD and Student's t-test was used *post-hoc* to ask which treatment levels account for differences in response to selection.

Table 2.4. Summary of selection sequences broken down by bad year frequency. Since some treatments have more bad years, they had higher number of years even though the entire duration of each selection line treatment was the same.

	Year Number													
Selection Line Treatment	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0%	G	G	G	G	G	G	G	G	G	G				
20%	**B**	G	**B**	G	**B**	G	G	G	G	G	G	G		
	G	G	G	G	G	G	**B**	G	**B**	G	**B**	G		
	G	G	G	**B**	G	G	G	**B**	G	**B**	G	G		
	G	G	G	G	G	G	**B**	G	**B**	G	**B**	G		
	G	G	G	G	G	**B**	G	**B**	G	**B**	G	G		
30%	**B**	G	**B**	G	G	G	G	G	G	G	**B**	G	**B**	G
	G	**B**	G	**B**	G	G	**B**	G	G	G	G	**B**	G	G
	B	G	**B**	G	G	**B**	G	**B**	G	G	G	G	G	G
	G	G	G	G	**B**	G	**B**	G	**B**	G	**B**	G	G	G
	G	G	G	G	G	G	**B**	G	**B**	G	**B**	G	**B**	G
40%	**B**	G	**B**	G	G	**B**	G	G	**B**	G	**B**	G	**B**	G
	G	**B**	G	**B**	G	G	**B**	G	**B**	G	**B**	G	**B**	G
	B	G	G	**B**	G	**B**	G	G	**B**	G	**B**	G	**B**	G
	B	G	**B**	G	**B**	G	**B**	G	**B**	G	G	G	**B**	G
	B	G	**B**	G	**B**	G	**B**	G	**B**	G	**B**	G	G	G
50%	**B**	G	**B**	G	**B**	G	**B**	G	**B**	G	**B**	G	**B**	G

2.3 Results

Analysis of covariance (Table 2.5a) reveals that the covariate, bad year frequency, is a significant positive predictor of evolved dormancy fraction (R^2=0.294), whereas neither genetic cross nor the interaction between bad year frequency and genetic cross show significant effects. Eleven of the twelve genetic crosses, including the mass mating cross, show evolution of increased dormancy with higher frequency bad years (Figure 4).

An analysis of covariance using net change in dormancy—the difference between the final dormancy fraction and the estimate of original dormancy for each cross—as the response variable corroborates the results. Bad-year frequency treatment and of genetic cross have significant effects on net change in dormancy fraction but, again, there is no evidence of heterogeneity of slopes among crosses (Table 2.5b). Six out of the twelve crosses did exhibit a negative net change in dormancy for each bad year frequency, but similar to the final dormancy fraction, eleven of the twelve genetic crosses show an increasing positive relationship between net change in dormancy and frequency of bad years (Figure 5).

Table 2.5. Analysis of covariance of the effect of the frequency of "bad years" on the evolution of a) final dormancy fraction and b) net change in dormancy fraction in twelve genetic crosses of *Neurospora crassa*. Cross is a random effect, and the bad years effect, which ranged from 0% to 50% in five selection lines, is a continuous covariate.

Trait	Source	Mean Squares	DF	F Ratio	p value
a) Final Dormancy Fraction					
	Bad Years	0.2653	1	8.4884	0.0049
	Cross	0.02490	11	0.7972	0.6421
	Cross*Bad Years	0.02377	11	0.7604	0.6771
b) Net Change in Dormancy					
	Bad Years	0.2653	1	8.4884	0.0049
	Cross	0.08762	11	2.8053	0.0049
	Cross*Bad Years	0.02377	11	0.7604	0.6771

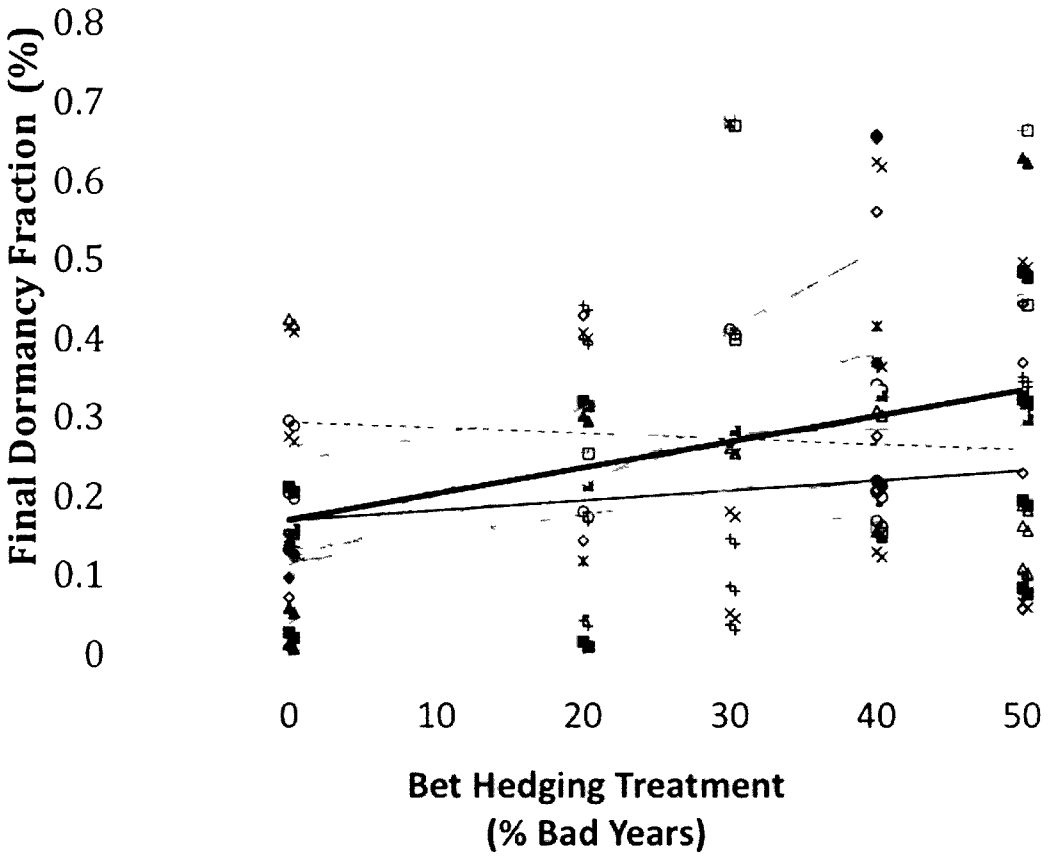

Figure 4. Final, post-selection dormancy fractions plotted over the five selection treatments differing in proportion of bad years. Thin lines represent regressions fitted for each of the twelve crosses: eleven exhibited positive slopes (solid) and one a negative (dashed) slope. The bold line represents the overall positive and significant (Table 2.5a) effect of bad years on the evolution of dormancy fraction.

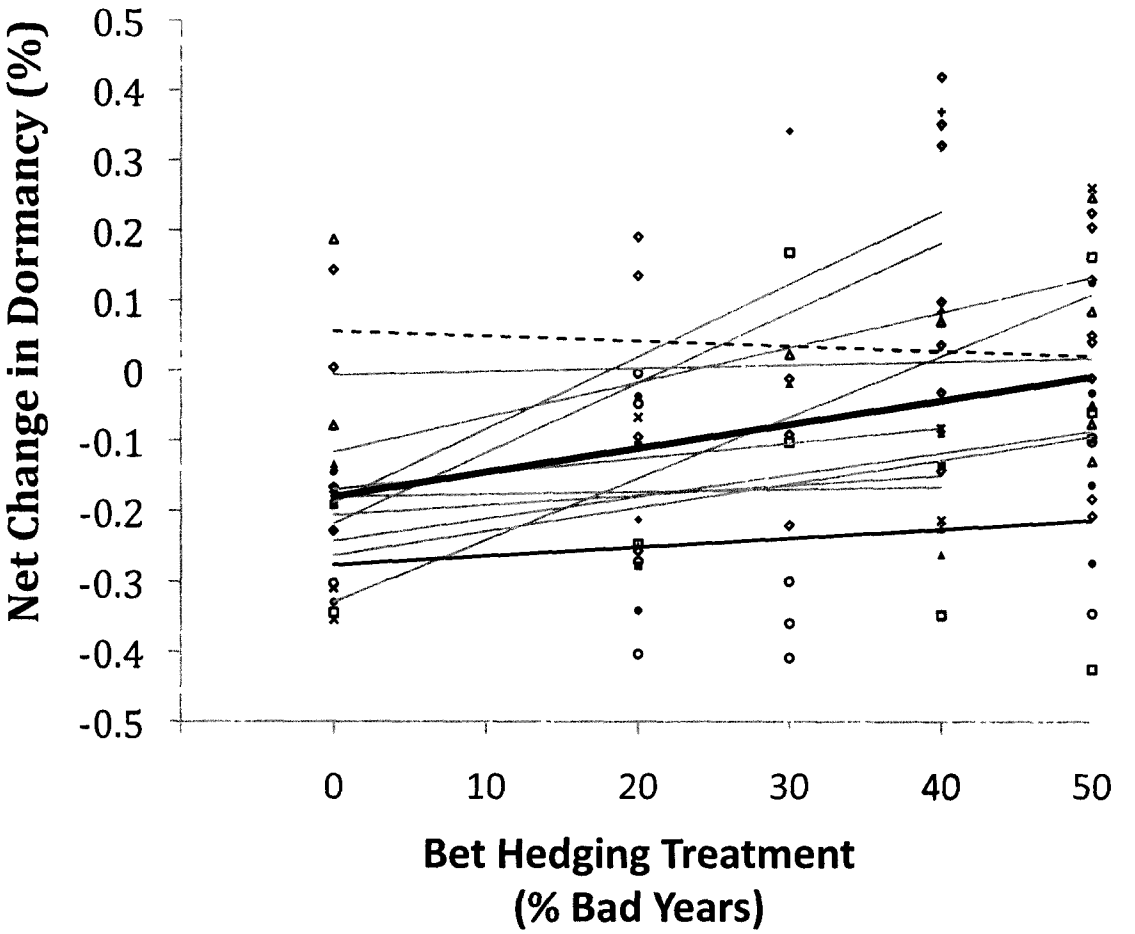

Figure 5. Net change in dormancy fractions plotted over the five selection treatments differing in proportion of bad years. Thin lines represent regressions fitted for each of the twelve crosses: eleven exhibited positive slopes (solid) and one negative (dashed) slope. The large bold line represents the overall positive and significant (Table 2.5b) effect of bad years on the evolution of dormancy fraction. Net change in dormancy was calculated by subtracting the average dormancy fraction of the initial mean dormancy of each cross prior to selection from each dormancy fraction of the same cross that was subjected to each selection treatment regime.

A one-way analysis of variance, treating the frequency of bad year effect as a nominal variable, detects significant differences among bad year treatments for both evolved final dormancy fraction and net change in dormancy fraction (Table 2.6), corroborating the trends observed when treating bad year as a covariate. Post-hoc analyses (Table 2.7) reveal most notably that the 0% bad-year selection treatment is consistently different from the 40% and 50% treatments by the Student's-t criteria, and from the 40% treatment by the more conservative Tukey's criteria.

Genetic crosses do not occur in sufficient numbers across all bad-year treatment levels to allow a two-way factorial analysis to be performed. However, an ANOVA using bad-year treatments collapsed into two year-type categories—0 to 20% bad years, and 30 to 50% bad years—shows a highly significant effect of year-type category ($df=1$; $F=9.066$; $P=0.0075$), and no evidence of either an effect of cross ($df=11$; $F=0.391$; $P=0.933$) or an interaction between bad year category and genetic cross ($df=11$; $F=1.091$; $P=0.383$) on evolved dormancy fraction. Qualitatively identical results are obtained for net change in dormancy fraction as the response variable.

Table 2.6. One-way analyses of variance for evolved dormancy fraction and net change in dormancy fraction in response to the proportion of bad years treatment.

Trait	Source	DF	Mean Sums of Squares	F Ratio	p value
Final Dormancy Fraction	Bad Years	4	0.085622	2.9011	0.0267
	Error	82	0.029513		
	Total	86			
Net Change in Dormancy Fraction	Bad Years	4	0.106018	2.9017	0.0267
	Error	82	0.036536		
	Total	86			

Table 2.7. Evolved dormancy and net change in dormancy fraction for each bad year treatment level. Means not connected by the same letter are significantly different at α=0.5 according to post-hoc Student-t and Tukey's HSD analyses from the one-way ANOVA (Table 2.6).

Trait	Bad Year Treatment	Mean	SE (±)	Student's t	Tukey's HSD
Final Dormancy Fraction	0	0.16056808	0.04049237	A	A
	20	0.2151302	0.04294864	AB	AB
	30	0.28544696	0.05179801	AB	AB
	40	0.32997567	0.04049237	B	B
	50	0.29929615	0.03506742	B	AB
Net Dormancy Change	0	-0.1672699	0.4505321	A	A
	20	-0.1421234	0.04778615	AB	AB
	30	-0.0876687	0.05763227	ABC	AB
	40	0.0189661	0.04505321	C	B
	50	-0.0348931	0.03901723	BC	AB

2.4 Discussion

Natural environments are stochastic (Halley 1996; Gonzalez and Descamps-Julien, 2004; Wilbur and Rudolf, 2006), and bet-hedging theory may thus be central to assessing evolutionary response in natural populations (Simons, 2009). Yet empirical tests of bet hedging are rare; no test has been devised to explicitly distinguish between the maximization of the geometric- vs. the arithmetic-mean fitness, even for propagule dormancy—the best-known example of diversification bet hedging. This study provides experimental evidence consistent with the predictions of the geometric-mean principle.

Results of artificial fluctuating selection on dormancy fraction are consistent with the predictions of Cohen's (1966) model of bet hedging. When subjected to selection sequences differing in unpredictable occurrence of "good years" and "bad years", dormancy fraction in ascospores of *Neurospora crassa* qualitatively fits the frequency of occurrence of "bad" years. The results are robust in that they concur with the predictions of Cohen's model by several criteria. First, the evolutionary trend in dormancy across the different bad-year selection lines is consistent for the two response variables, final dormancy fraction and net change in dormancy fraction. Second is the consistent difference between low (0%, 20%) and high frequency (40%, 50%) bad-year treatments in post-hoc analyses. Third, the rank order of evolved dormancy fractions is in almost perfect agreement with bad-year frequency, with only a nonsignificant and minor (3% difference in dormancy) counter-trend for the 40% and 50% bad-year treatments. Fourth, net change in dormancy fraction occurred both upward and downward, and in the predicted direction: although change in the 50% bad-year treatments was nonsignificant,

the mean dormancy fraction for the 40% increased, and that of the 0% and 20% lines decreased significantly during the selection experiment. Finally, the observation of agreement in evolutionary trends in final dormancy fraction across eleven of twelve genetic crosses cannot be reasonably explained by chance.

If selection maximized the arithmetic-mean fitness, a high germination fraction would be consistently favoured regardless of selection treatment, and thus there would be no difference among selection lines with respect to dormancy fractions. A slope of zero is thus predicted by the arithmetic-mean principle. However, this hypothesis of zero slope was rejected statistically by the ANCOVA, which demonstrates covariance between dormancy fraction and frequency of bad years, as indicated by the bold line in Figure 4. Furthermore, the evolutionary response in dormancy fraction among genetic crosses was consistent (Figure 4).

The results for final dormancy fraction are corroborated by those for net change in dormancy. The difference between dormancy in the unselected crosses and the selected crosses demonstrates that selection acted on the geometric-mean fitness. While there was an effect of cross, there was no statistical difference among slopes of crosses, and the overall trend was positive with respect to bad year frequency. The magnitude of their net change was almost in perfect agreement with those predicted by the bad year treatments. Taken together, the results are in line with the predictions of Cohen's (1966) model, which demonstrates that selection acts to increase the geometric-mean of fitnesses across generations, and not average or expected fitness within generations.

Although the experimental approach taken here eliminates many of the problems inherent to observational studies, confidence in the present results requires a closer examination of several potential difficulties. First, an experimental study can show that fluctuating selection results in bet hedging, but it cannot address the important question of how widespread bet hedging traits are in natural populations in the field. Second, although fourteen generations proved sufficient to produce a measureable evolutionary response, it is not known whether dormancy fraction reached an evolutionary equilibrium. For this reason, a quantitative test of predictions of Cohen's model may be unreliable. For example, the 0% bad-year treatment line evolved a reduced (16%) but nonzero dormancy fraction; similarly, the 40% and 50% bad-year treatments evolved 33% and 30% dormancy respectively, which imperfectly fits the 50% quantitative prediction and thus do not suggest a difference in evolutionary response between these two treatments. Results suggest that selection acts on the geometric-mean fitness and that bet hedging has evolved, but further examination of the time course of dormancy evolution and an experiment extended beyond fourteen generations would be necessary to allow a test of the quantitative fit of evolved dormancy fraction to predictions. As it stands, we established a qualitative fit, not a quantitative fit between the observed and predicted dormancy fractions.

Third, it is conceivable that "predictive germination" based on imperfect cues (Tielbörger and Valleriani, 2005; Ergon, 2007) influenced final germination fractions, in that lines encountering a good year in the final generation were more likely to germinate during the test. This possibility was eliminated by taking spores of all selection lines through a generation under "good" conditions prior to the final germination test. Fourth,

the late addition of the 50% bad-year treatment by splitting off from the 40% bad-year treatment introduces some non-independence between the two treatments, even if germination tests were performed independently. However, the complete removal of the 50% selection line from the ANCOVA—a conservative test—does not qualitatively alter the outcome for either dormancy fraction or net change in dormancy. Finally, the percent bad year selection lines were not evenly distributed from 0% to 50%, and not all genetic crosses were represented in all selection lines because of their loss during the course of the experiment. A factorial mixed-model ANOVA using two year-type groupings (low: 0-25% bad years, high: 30-50% bad years) shows a highly significant effect of year type group ($P=0.0075$), no effect of genetic cross ($P=0.933$), and no effect of their interaction ($P=0.383$) on the evolution of dormancy, and thus corroborates the ANCOVA results. Furthermore, the consistency among genetic crosses in their evolutionary response to selection treatments shows that it is improbable that results were biased due to loss of genetic crosses.

Conclusive evidence for bet hedging is difficult to obtain in the field, because it requires demonstrating that the proposed bet-hedging trait acts to maximize geometric-mean fitness over generations. The most common form of evidence for diversification bet hedging is the observation of trait variance coupled with a description or quantification of environmental variance. The strongest observational studies show a correlation between the expression of bet hedging and measures of unpredictable selection that differ among populations. For example, Clause and Venable (2000) showed that dormancy fractions in the desert annual *Plantago insularis* closely match expectations for historical rainfall; specifically, that dormancy is high in more xeric

environments. However, they concluded that phenotypic plasticity accounts for more of the variation in germination fractions than does evolved population differences. Also in the field, Donohue and colleagues (2005) demonstrated that natural selection favours diversification in the timing of seed germination in *Arabidopsis thaliana.* By using a modeling approach to quantifying fitness effects of dormancy in a multi-year study, Evans *et al.* (2007) showed that in variable environments, the long-term growth rate is higher in individuals exhibiting dormancy than in those showing no dormancy. Venable's (2007) exhaustive 22-year study of 10 desert annuals provides evidence in support of bet-hedging theory by showing that individuals with high variance in reproductive success also have higher dormancy fractions.

Testing at the individual level is vital for empirical evaluations of bet hedging (Seger and Brockmann, 1987; Rees *et al.*, 2009; Simons, 2006, 2009; Childs *et al.*, 2010), which can add considerable challenges to empirical work. For example, individuals in field conditions much less in nature are not necessarily demographically homogeneous (Tuljapurkar *et al.*, 2003), which means that measuring traits at the population level would prove misleading; however, measuring traits at the individual-level of a structured population is onerous. Another difficulty is that there is no "correct" time-scale for empirical investigation of the evolution of bet hedging because environments are increasingly variable over longer time scales (Boyce *et al.*, 2006; Wilbur and Rudolf, 2006; Childs *et al.,* 2010), leading to the expectation that bet-hedging traits may occur at any level of biological organization (Simons, 2002). An additional problem common to all observational studies is that correlations between various life-history traits and demographic components make it difficult or impossible to link fitness effects to specific

traits. Experimental manipulation may be used to overcome these difficulties, and such an approach was used to show that fluctuating natural selection in the field can account for the evolution of diversification in germination behaviour in *Lobelia inflata* (Simons, 2009).

Experimental study of bet hedging can build upon field evidence by employing simple model systems that can control for the many confounding and interacting variables present in observational study in the field. Ratcliff and Denison (2010) observed that the bacterium *Sinorhizobium meliloti* generated individual-level phenotypic diversification by producing two divergent types of daughter cell when starved. Veening *et al.* (2008) demonstrate that 'phenotypic switching' in bacterial cells is the result of differential gene expression pattern known as bistability, which can be interpreted as a bet-hedging trait. Beaumont *et al.* (2009) used the bacterium *Pseudomonas fluorenscens* to select for stochastic phenotypic switching in colony morphology. This pioneering work demonstrates that a proposed bet-hedging trait can evolve in bacterial systems, but it does not provide evidence for bet hedging, or specifically address whether selection maximizes the geometric mean fitness, because stochastic phenotypic switching was directly selected for.

A focus on Cohen's (1966) model, and the use of a simple model system provides the key advantages necessary for a direct test of the geometric-mean principle. Dormancy is unambiguously a diversifying bet hedging trait (Childs *et al.*, 2010) in that its evolution is a straightforward prediction of the geometric-mean principle (Dempster, 1955). The life history of *N. crassa* that includes a dormant propagule, combined with the well-developed protocols for its usage in laboratory settings (Davis, 2000), allowed

for a direct test of Cohen's predictions. This experimental system allowed the definition of discrete "good" and "bad" environments, and control of the sequences of these environments consistently over the experiment. This experimental approach ensured that environmental unpredictability was the source of any evolutionary change in dormancy fraction, and that evolution was not a result of direct selection for dormancy, the trait assumed to be bet hedging. Instead, to directly ask whether dormancy evolves as bet hedging, selection was effected through repeated representative sampling of all spores, both dormant and non-dormant, as founders for each subsequent generation under fluctuating conditions. This empirical test thus differs from others because it gives compelling evidence that selection maximizes the geometric-mean fitness.

Although dormancy is known across kingdoms (Sussman and Halvorson, 1966; Clutter 1978; Baskin and Baskin, 1998), work on the genetic and mechanistic basis of the expression of dormancy as a bet-hedging trait is deficient. Along with continued field work on bet hedging (Claus and Venable, 2000; Evans *et al.*, 2007; Venable, 2007), simple model systems like *N. crassa* could be used to more specifically explore the evolutionary dynamics of bet hedging traits such as dormancy in experiments over longer time scales to provide greater empirical weight to, and understanding of bet hedging. In addition, with the extensive accumulated knowledge of *N. crassa,* including its sequenced genome (Galagan *et al.*, 2003), a genetic understanding of bet hedging could give greater insight into how organisms express conservative or diversifying bet-hedging traits in variable environments.

In this thesis, I have provided significant evidence that fluctuating selection acts to maximize the geometric-mean fitness and by extension, that its outcome is the

evolution of bet-hedging traits. These results highlight the larger issues of the potential general importance of bet hedging, how the geometric-mean principle might affect our understanding of trait optimality, and suggest that bet hedging should not be considered a "special case" of evolution (Simons, 2002). Furthermore, bet hedging may explain differences among species in persistence in the face of rapid environmental change (Simons, 2011). These issues cannot be resolved by experimental evolutionary studies in the lab, however, because the importance of the geometric-mean fitness principle depends on the degree of environmental unpredictability in nature, and on the relative importance of other responses to environmental change such as adaptive tracking and adaptive phenotypic plasticity (Via and Lande, 1985; Schlichting and Pigliucci, 1998; Bell and Collins, 2008; Simons, 2011). Clearly, the geometric-mean principle is of evolutionary importance, and further work is needed to elucidate the extent to which bet hedging evolves in the face of natural environmental stochasticity.

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