Asynchronous spore germination in isogenic natural isolates of *Saccharomyces paradoxus*

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Abstract

Spores from wild yeast isolates often show great variation in the size of colonies they produce, for largely unknown reasons. Here we measure the colonies produced from single spores from six different wild *S. paradoxus* strains. We found remarkable variation in spore colony sizes, even among spores that were genetically identical. Different strains had different amounts of variation in spore colony sizes, and variation was not affected by the number of preceding meioses, or by spore maturation time. We used time-lapse photography to show that wild strains also have high variation in spore germination timing, providing a likely mechanism for the variation in spore colony sizes. When some spores from a laboratory strain make small colonies, or no colonies, it usually indicates a genetic or meiotic fault. Here, we demonstrate that in wild strains spore colony size variation is normal. We discuss and assess potential adaptive and non-adaptive explanations for this variation.

Introduction

Offspring from the same parents often vary because of mutation, recombination, and segregation. These kinds of random genetic variation underlie evolutionary adaptation by natural selection. The rate of random variation is itself expected to be selected (Barton and Charlesworth 1998, Fisher 1930, Kimura 1967), and there is some evidence that both mutation rate (Sniegowski, et al. 1997) and recombination rate (see references in Michod and Levin 1988, Otto and Lenormand 2002) are optimized by natural selection (but see e.g. Clune, et al. 2008, Furió, et al. 2005). However, offspring can also vary even when they are genetically identical and share a common environment. Such variation can potentially increase fitness: for example, microbial cells that divide more slowly may be better able to survive environmental stresses than their faster growing clone mates (Balaban, et al. 2004, Levy, et al. 2012). When such stochastic variation is the target of natural selection in unpredictable environments, it is known as "bet-hedging" (Cohen 1966, Simons 2011). Some forms of variation among genetically identical individuals may be heritable from one generation to the next, for example chromatin patterns that affect gene expression. Such variation is usually described as "epigenetic", and is itself subject to natural selection (Daxinger and Whitelaw 2012, Heard and Martienssen 2014).

Saccharomyces yeast can reproduce both asexually and sexually, and the life cycle allows many forms of variation among offspring to occur. It is thought that most reproduction in *Saccharomyces* occurs by diploid mitosis (Greig and Leu 2009) but if starved some diploids enter meiosis, each producing a tetrad of four haploid spores (two of each mating-type) enclosed in an ascus and joined by inter-spore bridges (Coluccio and Neiman 2004). These dormant and resistant spores germinate in response to nutrient availability, and they can fuse with other germinated haploids of the opposite mating type, either from the same

tetrad (a form of self-fertilisation we will refer to as 'within-tetrad mating') or from another tetrad (we will call this 'between-tetrad mating'). Unmated haploids can reproduce by haploid mitosis and can switch mating type, enabling mating and diploid formation within a clone derived from a single haploid cell (we will call this extreme form of self-fertilisation 'autodiploidisation'). Genetic variation in the yeast life cycle is thus generated by mutation and by the sexual processes of meiosis and syngamy, but non-genetic variation is also possible at every point in the cell cycle, so that even clonal cultures are expected to contain cells in different states. Variation in *Saccharomyces* has been demonstrated in a variety of traits, e.g. cell size (Alberghina, et al. 1998, Spor, et al. 2008), cell surface proteins (due to position-effect variegation and gene silencing; Aparicio, et al. 1991, Halme, et al. 2004), and growth rate (due to prion effects; Halfmann, et al. 2012).

Here, we investigate another striking, but understudied, example of phenotypic variation among genetically identical yeast cells: spore germination asynchrony. Spore germination in yeast occurs when spores sense glucose. In laboratory strains of *S. cerevisiae*, commitment to germination usually occurs about 15 minutes after transfer to rich medium, and mitosis begins after about 4 hours (Joseph-Strauss, et al. 2007). We noticed that when we dissect isogenic haploid spores from the meiotic tetrads of wild *S. paradoxus* isolates, many fail to produce colonies, and the colonies that do form often vary greatly in size. Other researchers who work on wild yeast have made similar observations, but to date no formal description of this phenomenon has been published. To quantify this colony size variation in wild strains, we measured the sizes of colonies produced after isogenic spores are placed simultaneously on agar (1. in graphical abstract). We show that colonies produced by vegetative cells have much less variation in size than colonies produced from isogenic spores, suggesting that variation in spore colony size was due to differences in germination timing rather than to differences in vegetative growth rate (2. in graphical abstract). To quantify

variation in spore germination timing, we used a microscope to make time-lapse photographs of germinating spores (3. in graphical abstract). We then test two treatments that have been previously suggested to improve the synchrony of germination in wild yeast: multiple rounds of meiosis (Naumov, et al. 1995), and aging of tetrads before germination (Greig 1999) (4. and 5. in graphical abstract). We discuss the evolutionary implications of spore germination asynchrony, and show that spores from the same tetrad (even when dissected out of their ascus onto different parts of an agar plate) are more likely to germinate at the same time than isogenic spores from another tetrad, which is inconsistent with spore germination asynchrony being a strategy to increase between-tetrad mating (outbreeding) but is consistent with it being a strategy for biological bet-hedging in an unpredictable germination environment.

Materials and Methods

Strains and sporulation protocol

We used six wild *S. paradoxus* strains, each isolated from the bark of a different oak tree in Plön, Germany, in October 2010 (Kowallik, et al. 2015). Each primary isolate was allowed to sporulate for two days at room temperature on sporulation medium (2% potassium acetate, 0.22% yeast extract, 0.05% glucose, 0.087% complete amino acid mix, 2.5% agar; (Amberg, et al. 2005)). The resulting tetrads were treated with 10 µl zymolyase (100 U/ml) for 30 minutes at room temperature, then 500 µl of sterile water was added, and the tetrads were incubated for at least 30 minutes more before spores were dissected out of the digested tetrads onto YPD plates [2% dextrose, 2% bactopeptone, 1% yeast extract, 2.5% agar] using a Singer MSM 400 micro-manipulator. Plates were incubated for two days at 30°C and then a single colony derived from a single spore was selected at random from each strain, propagated on YPD and frozen for future use. All six of these strains (Plön2 through Plön7) could themselves sporulate, showing that they had undergone autodiploidisation from the

haploid spores from which they were derived. They were therefore perfectly homozygous except at the *MAT* locus. We also used the standard laboratory *S. cerevisiae* strain Y55 (McCusker and Haber 1988), which was previously derived from an autodiploidised haploid, for comparison.

Variation in the size of colonies produced by isogenic spores

To measure the variation in the size of colonies produced by single spores (hereafter "spore colonies") from each strain, we began by placing a sample of digested tetrads of each Plön2 through Plön7 at the edge of a YPD plate and then dissecting 25 spores (from 6 complete tetrads, plus a single spore from a seventh tetrad), and placing them 12 mm apart in a 5 x 5 grid (Figure 1). Ten such grids were prepared for each of the wild strain (i.e. 250 spores per strain). Grids were then incubated at 25°C for 3 days, and the number of colonies visible to the naked eye was counted. The sizes of the visible colonies were determined by electronically scanning the YPD agar plates and using ImageJ (Abramoff, et al. 2004) to measure the area of the colonies ('Analyze Particles' option with size [200 - Infinity], and circularity [0.4-1.0]). We calculated the radii of the colonies by treating them as perfect circles and determined the standard deviation of the radii as a measure of the variation in spore colony size (point 1. in graphical abstract; Table 1). We then incubated the plates for a further 14 days and again recorded the number of visible colonies.

Variation in the size of colonies produced by isogenic vegetative cells

To test if size variation in spore colonies is due to differences in the intrinsic vegetative growth rates of cells derived from different spores, we compared the sizes of colonies produced from spores with the sizes of colonies produced from single vegetative cells

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(hereafter "vegetative cell colonies"). For each of seven strains (Plön2 through Plön7, plus Y55), we dissected ten tetrads onto a YPD plate and incubated the seven plates containing 40 spores each at 25°C for 3 days to produce spore colonies. We then selected ten pairs of small and large spore colonies from each plate (i.e. ten pairs of large and small colonies per strain) and measured their sizes in ImageJ as described above (Figure 2a). For each pair of colonies, we streaked vegetative cells from one colony on one side of a new YPD plate, and vegetative cells from the other colony on the other side of the same plate, (so that variation in plates would not affect differences in vegetative cell colony sizes between each strain in a pair; Figure 2b). These 70 plates (1 pair per plate, ten pairs per strain) were incubated at 25°C for 3 days to produce vegetative cell colonies. The mean vegetative cell colony size of streaked cells was measured as the average size of three representative vegetative cell colonies from each side of the plate (point 2. in graphical abstract). These colonies were selected blind by a lab member who was unaware of the identity of the strains or the purpose of this experiment. We then calculated the absolute size difference between small and large spore colony in each pair, and the absolute size difference between average vegetative cell colony sizes in each pair, and compared these differences in paired t-test across all ten pairs from all 7 strains (i.e. n=70 pairs; Table S1).

Variation in germination timing of isogenic spores

To quantify variation in spore germination timing we first sporulated all six homozygous wild *S. paradoxus* strains plus the *S. cerevisiae* strain Y55, and prepared them for dissection as described above. We dissected 80 spores (from 20 tetrads) from each strain into a 8 x 10 grid within a single microscope field of view (approximately 0.086 mm x 0.078 mm). The seven grids, one from each strain, were incubated at 25° C, and we photographed the grid of spores every five minutes for 48 hours. Periodically, germinated cells were removed so that

they did not form colonies that could interfere with neighbouring ungerminated spores. The photographs were then examined to determine the exact time of germination, as determined by the first appearance of a bud (point 3. in graphical abstract; Table 2, Table S2). Spores germinating after 48h were excluded from this analysis.

The effect of spore age on colony size variation

To test the hypothesis that variation in spore colony size is affected by spore maturation time (point 4. in graphical abstract; Table S3), we sporulated each homozygous wild strain (Plön2 through Plön7) as described above but left the spores on the sporulation medium for 2, 9, 16, 21, 28, 30, or 37 days before measuring the variation in spore colony size. Two 5 x 5 spore grids were prepared for each strain at each time point (i.e. 50 spores per strain on 2 YPD plates), except for the 9-day maturation period, where four grids were used per strain (100 spores on 4 plates total). Grids were incubated at 25°C for 3 days, and the spore colony sizes of visible colonies were measured as described above.

The effect of preceding meiotic cycles on colony size variation

To test the hypothesis that repeated meiosis affected spore colony size variation, we first dissected 50 spores from each wild strain (Plön2 through Plön7) onto two 5 x 5 grids each, and incubated for 3 days at 25C (as described above in *Variation in the size of colonies produced by isogenic* spores). We then picked cells from the largest spore colony from each strain onto sporulation medium. We sporulated the cells derived from the largest colony from each strain by incubating them at 25°C for 2 days, and then we repeated the cycle, by dissecting 50 of the resulting spores from each strain, picking the largest colony from each strain, and sporulating them again (point 5. in graphical abstract; Table S4). We completed

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this cycle 13 times in total. After each cycle, variation in spore colony sizes was measured as described above (*Variation in the size of colonies produced by isogenic spores*).

Results

Variation in the size of colonies produced by isogenic spores

Colony sizes produced by isogenic haploid spores varied greatly in size in all strains (Table 1). On average, the radii of the smallest and largest colonies per strain differed by 1.11mm ($\pm$ 0.074), which is substantial considering the average radius of colonies was 0.93mm ($\pm$ 0.21).

We were able to reject the null hypothesis that the average spore colony size did not differ among strains (one-way ANOVA of strain affiliation on colony size: $F_{5,1078} = 13.6$, $p <$ 0.001; Table 1). We were also able to reject the null hypothesis that colony size variances did not differ among strains (Levene's test for unequal variances: $F_{5,1078} = 13.5$, $p < 0.001$). We were also able to reject the null hypothesis that differences among strains in the numbers of visible and invisible (i.e. ungerminated) colonies they produced after three days were due to chance (7x2 contingency table: $\chi^2_5 = 66.45$, $p < 0.001$) and 14 days ($\chi^2_5 = 28.14$, $p < 0.001$; Table 1). While most spores successfully germinated (72% $\pm$ 0.1 across strains), many failed to produce visible colonies (Table 1). Across all strains, 72% of all spores had produced visible colonies after 3 days, and 83% had produced visible colonies after 14 days. Thus different strains produced differently sized colonies with different variances for colony size, and the number of visible colonies produced was contingent on the strain.

Variation in the size of colonies produced by isogenic vegetative cells

Even though genetically identical strains are commonly assumed to have identical vegetative growth rates, isogenic individuals can vary considerably in their division time. It is possible then that variation in vegetative growth rates after germination might explain the variation in

colony sizes that we see. To test this, we selected and measured pairs of large and small spore colonies from the same plate, and streaked both members of each pair on the same plate and measured the resulting vegetative cell colonies (Figure 2). We took the absolute size difference between the small and large spore colony in each pair, and the absolute size difference between vegetative cell colony sizes in each pair, and compared them as paired observations (Table S1). The absolute differences in vegetative cell colony sizes in each pair were much less than the absolute differences in spore colony sizes in each pair, which rejects the null hypothesis that the mean difference between the paired observations from the two groups is zero (two-tailed paired t-test including wild strains only $t_{59} = 20.12$, $p < 0.001$, two-tailed paired t-test including wild strains and Y55 $t_{69} = 15.71$, $p < 0.001$). We conclude that streaking vegetative cells from spore colonies so that they produced new vegetative cell colonies significantly reduced the size difference between paired colonies. These results are consistent with the hypothesis that the size differences among isogenic primary spore colonies were due to differences in spore germination times rather than vegetative growth rates.

Variation in germination timing of isogenic spores

We measured the time between placing spores on the surface of a plate and germination of individual spores, using time-lapse photography for 48h. Spores germinating after this were excluded from the analysis. We were able to reject the null hypothesis that differences in the variances in measured isogenic spore germination time among strains were due to chance (Levene's test for equal variances including wild strains and Y55 after log transforming data for normality: $F_{6,279} = 10.97$, $p < 0.001$; Table 2; Table S2; Figure 3), thus different strains had different amounts of variation in spore germination timing. Tukey test for multiple

comparisons showed that the variation in spore germination of all wild *S. paradoxus* strains significantly differed from that of the *S. cerevisiae* laboratory strain Y55.

We were able to reject the null hypotheses that the average time to germination did not differ among strains (one-way ANOVA of strain affiliation on germination timing, including wild strains only: $F_{5,204} = 30.26$, $p < 0.001$; wild strains and Y55: $F_{6,279} = 101.67$, $p < 0.001$), and that the proportion of spores that germinated within 48 hours did not differ among strains (including wild strains only: $\chi^2_6 = 58.38$, $p < 0.001$; wild strains and Y55: $\chi^2_6 = 144.78$, $p < 0.001$; Table 2). There were thus statistically significant differences between strains in the average time their spores took to germinate and in the proportion of spores that germinated.

The effect of spore age on colony size variation

We used linear regression to test whether spore age affected standard deviation in spore colony size. We were unable to reject the null hypothesis that the population correlation coefficient was zero (Pearson's correlation coefficient for Plön2 - Plön7, respectively: $r_{1,5} = -0.12$, $r_{1,3} = -0.74$, $r_{1,5} = -0.11$, $r_{1,3} = -0.13$, $r_{1,5} = -0.45$, $r_{1,3} = 0.17$; all $p > 0.152$; Figure 4; Table S3). Thus there is no support that spore maturation time affects variation in germination time, a hypothesis proposed previously (Greig 1999).

The effect of preceding meiotic cycles on colony size variation

A previous study suggested that variation in spore colony size or viability can be reduced by repeated meiosis and autodiploidisation, referred to as monosporic cloning (Naumov, et al. 1995). Naumov et al. reported that after monosporic cloning, spores germinated more synchronously and produced more equal sized colonies. We thus used linear regression to test whether the number of cycles of meiosis affected the standard deviation of spore colony size

in wild strains. However, we were unable to reject the null hypothesis that the population

correlation coefficient was zero (Pearson's correlation coefficient for Plön2 - Plön7,

respectively: $r_{1,11} = -0.2$, $r_{1,11} = -0.17$, $r_{1,11} = -0.38$, $r_{1,11} = -0.37$, $r_{1,11} = -0.32$, $r_{1,11} = 0.24$; all p

> 0.196; Figure 5, Table S4). Therefore there is no evidence that meiosis affects isogenic

spore germination time variation.

The distribution of germinated spores among tetrads

Because the ability to mate with gametes from the same tetrad depends on gametes from the

same tetrad germinating at similar times, we tested whether the germination of a spore

depended on whether or not other spores in the same tetrad had germinated (even when

spores were dissected out of their ascus onto different parts of an agar plate). For this, we

used the data set produced with time-lapse photography over the course of 48h. Spores

germinating after 48h were excluded here. We were able to reject the null hypothesis that the

distribution of germinated spores among the 20 tetrads from each wild strain is a Poisson

distribution (all $\chi^2_4 > 10.66$, p < 0.03; Table 2; Figure 6). All strains showed a more extreme

distribution, i.e. there were more tetrads with zero or four spores germinating, and fewer

tetrads with one, two, and three spores germinating, than expected by chance (Figure 6; Table

S5). This is consistent with spore germination being more likely in spores from tetrads that

produced other germinated spores, as expected if within-tetrad mating is favoured.

Because spores from the same tetrad were placed together in rows on the grid, it is

possible that the non-random distribution of germinated spores among tetrads described

above was due local differences in the germination environment in different parts of the plate.

We therefore also assigned spores into groups of four within columns, such that each plate

was divided into 20 "perpendicular quadruplets" of four spores that shared the same location

of the plate but came from different tetrads. We again tested the null hypothesis that the

distribution of germinated spores in perpendicular quadruplets is a Poisson distribution, and were unable to reject the null hypothesis for any strain (all strains: $\chi^2_4 < 3.99$, p > 0.41; Table 2; Table S6). This is consistent with the non-random distribution of germinated spores among tetrads being due to differences among the tetrads themselves rather than differences among the parts of the plate the spores from a tetrad occupy.

Discussion

The great variation in colony sizes produced from isogenic *S. paradoxus* spores is often noted by collectors of wild yeast and may be confused with spore inviability due to aneuploidy, genetic incompatibility, unmasking of recessive lethal alleles, or some meiotic problem. It has been mentioned anecdotally in the literature, but as far as we know there has been no formal description or investigation of this phenomenon. We found large variation in colony sizes produced by genetically identical spores grown under the same environmental conditions in six wild *S. paradoxus* strains. Streaking cells from differently sized spore colonies onto new growth medium resulted in evenly sized vegetative cell colonies, irrespective of the size of the spore colonies (Figure 2), consistent with spore colony size variation being caused by variation in germination timing rather than variation in vegetative growth rates. However, it is known that cells in the vegetative state are able to switch between slow-growing "persister" and fast-growing "non-persister" phenotypes (Levy, et al. 2012). It is therefore formally possible that the variation in spore colony size is actually due to variation in the composition of colonies, rather than variation in germination time, such that colonies with an initially high proportion of slow growers are smaller than those with an initially high proportion of fast growers. Competition within the colony and subsequent streaking the cells might select for fast growers, such that vegetative cell colonies become more similar in size than spore colonies. We have no evidence that variation in vegetative

cell growth contributes to the variation in spore colony size, but we have good evidence that spores germinate at different times, so we favour spore germination as the major mechanism underlying spore colony size variation.

Time-lapse photography from the moment spores were placed on growth medium to the exact time they germinated showed large variation of spore germination timing both within and between strains (Figure 3). Spores from wild *S. paradoxus* strains took longer to germinate and had much greater variation in germination time than spores from the standard *S. cerevisiae* strain Y55. Variation in spore colony size was not affected by the number of preceding meiotic cycles, nor was it affected by spore maturation time (Figure 4 and 5), contradicting previous studies suggesting that variation in spore colony size or viability can be reduced by repeated meiosis and autodiploidisation, referred to as monosporic cloning (Naumov, et al. 1995), or by allowing spores to mature for prolonged periods (Greig 1999).

The question remains: Why would germination asynchrony exist in wild strains of *Saccharomyces*? Spore germination in *Saccharomyces* requires fundamental changes in the morphology, metabolism, and physiology of the cell (Joseph-Strauss, et al. 2007). Given the extreme physiological differences between dormant spores and active cells, it is likely that variation in the transition between these states is subject to natural selection. This is supported by studies showing that differences in germination timing between species is a means for mate discrimination, contributing to the pre-zygotic isolation of *Saccharomyces* species (Murphy and Zeyl 2012). Thus, one may predict that germination timing is an important life history trait, which is under strong directional selection and has an optimum phenotype. It is therefore surprising to find such great variation both among genetically identical spores of the same strain and between genetically different strains of the same species.

We suggest three hypotheses for explaining the variation in germination timing among genetically identical spores. First, variation in germination timing is not an adaptation, but simply an inevitable consequence of stochastic noise in the complex environmental sensing systems that determine spore germination. This is the simplest and perhaps the most likely explanation, and should be the null hypothesis against which other, adaptive, explanations are tested. However the importance of germination as a life history trait and the ability of some strains to germinate much more synchronously than others suggest that spore germination asynchrony may be an adaptation. There is evidence in *S. cerevisiae* for significant heterogeneity in growth rates among genetically identical individuals within an environment, and strains isolated from different environments have different levels of heterogeneity (Ziv, et al. 2013). This supports the idea that variation in spore germination, requiring major transcriptional changes in response to the environment, may indeed be maintained by selection.

A second hypothesis is that germination asynchrony is a mechanism allowing a strain to modulate its rate of within-tetrad mating according to its fitness in the local environment. We have previously shown that more fit strains are more likely to mate within the tetrad, and we suggest that this might be an adaptation for preserving locally adapted gene complexes (Miller and Greig 2014). A spore that germinates at a different time from the other spores in its tetrad will be unable to mate with them, and must therefore either mate with a germinated spore from another tetrad or autodiploidise. Even if more than one spore in the same tetrad germinates at the same time, both spores must be opposite mating types in order to mate. Thus between-tetrad mating is likely to increase whenever a tetrad contains both germinated and ungerminated spores as a result of germination asynchrony. The differences in germination asynchrony among strains may therefore be due to their different degrees of adaptation to the laboratory media and conditions that we used, with less fit strains being

more asynchronous and more likely to mate with cells from other tetrads. Consistent with this, we found that the laboratory *S. cerevisiae* strain Y55, which is presumably well adapted to lab conditions, germinated more synchronously than most wild *S. paradoxus* strains.

A third hypothesis is that germination asynchrony is a form of adaptation known as biological bet hedging. Stochastic variation in a trait can be selected if it increases geometric mean fitness in an unpredictable environment. Verified examples of adaptive biological bet hedging are rare (Simons 2011) but there is recent evidence for bet hedging as an adaptation to fluctuating selection in bacteria (Beaumont, et al. 2009, Johnson and Levin 2013), fungi (Graham, et al. 2014), and *S. cerevisiae* (Levy, et al. 2012). In *Staphylococcus aureus*, for instance, stochastic variation in growth rate allows slow growing cells to survive antibiotic treatment killing their faster-growing clone mates, and such "persisters" can give rise to genetically distinct antibiotic-resistant strains (Johnson and Levin 2013). The classic theoretical example of biological bet hedging is variation in seed germination of annual plants, which can preserve ungerminated seeds for the future in case unpredictable environmental changes destroy already germinated seedlings (Cohen 1966). Like plant seeds, *Saccharomyces* spores are resistant to various environmental stresses that kill vegetative cells (including organic solvents, digestive enzymes, heat, and desiccation), so bet hedging is an attractive explanation for the germination asynchrony we describe.

To distinguish between the latter two adaptive explanations for spore germination asynchrony, increasing between-tetrad mating and biological bet-hedging, we tested whether the distribution of germinated spores among tetrads after 48 hours of germination opportunity was random or not (spores germinating after 48h were excluded). We assume that all spores can germinate, but some may delay their germination as an adaptation, either to increase the probability of between-tetrad mating, or to bet-hedge. Between-tetrad mating is more likely to occur when a spore germinates at a different time to the other spores in the same tetrad but

at the same time as other spores in other tetrads. So to maximize between-tetrad mating, spore germination should be asynchronous relative to the other spores in the tetrad, rather than relative to the other spores in the whole population. If spore germination asynchrony was an adaptation to promote between-tetrad mating, we might find more tetrads containing both germinated and ungerminated spores, and fewer tetrads in which all four spores germinated than we would expect if germination was random with respect to tetrads, i.e. if each spore had the same probability of germinating, independent of whether or not other spores in the same tetrad germinated. But in fact we found the opposite: there were more tetrads in which all four spores germinated than would be expected from the Poisson distribution that would be generated if germination was random. Thus spore germination asynchrony does not increase between-tetrad mating as much as it would if the ungerminated spores were randomly distributed among tetrads. This is consistent with germination asynchrony instead being a mechanism for bet hedging, allowing the spores in some tetrads to germinate but not those in others, whilst maintaining synchrony of spores within tetrads in order to promote within-tetrad mating as much as possible.

Whilst bet-hedging is an attractive explanation for spore germination asynchrony, we should bear in mind the advice of George Williams: "Evolutionary adaptation is a special and onerous concept that should not be used unnecessarily, and an effect should not be called a function unless it is clearly produced by design and not by chance" (1966). Ideally we should show that stochastically varying natural conditions exist in which one condition favours early germination, and another favours later germination, and that stochasticity in germination improves geometric mean fitness compared to either fixed phenotype (Simons, 2011). This is a tall order: we know so little about yeast natural history or ecology that we can only guess as to what the costs and benefits might be. We call spores "resistant", but it is not even clear what natural hazard they resist – presumably not the chloroform that many researchers use to

kill vegetative cells. It is striking, though, both from these results and from anecdotal observations, that wild *S. paradoxus* has much more asynchronous spore germination than laboratory *S. cerevisiae*. However, it might be that laboratory strains have been artificially selected to have more synchronous germination, perhaps because it makes tetrad analysis easier. It will be interesting to see whether wild *S. cerevisiae* spores are as asynchronous as wild *S. paradoxus.* It is also worth noting that even in the laboratory strain Y55, there were more tetrads in which all four spores germinated than would be expected by chance, consistent with the hypothesis that germination asynchrony is an adaptation. However, non-adaptive mechanisms could also produce this behaviour, for example spore size affects germination time and the spores within a tetrad might be more similar in size than the spores in different tetrads, even when they are all genetically identical, giving the observed pattern of within-tetrad synchrony and between-tetrad asynchrony (Smith, et al. 2014). Finally, we should remember that laboratory conditions are probably very different from natural conditions, and yeast may be very poorly adapted to the lab. It is therefore to be expected that some phenotypes or behaviours that are expressed in the lab may be irrelevant or may never even occur in the wild. Germination asynchrony, like other putative "bet-hedging traits", might be a lab artifact.

Conclusion

Here we show that spores from wild *S. paradoxus* strains tend to germinate asynchronously on standard laboratory media, even when they are genetically identical, producing colonies that differ greatly in size, in contrast to the identical colonies observed when tetrads from laboratory *S. cerevisiae* are dissected. Neither the mechanism underlying this variation, nor the evolutionary reason for it is yet known, but the phenomenon is an attractive subject for future study.

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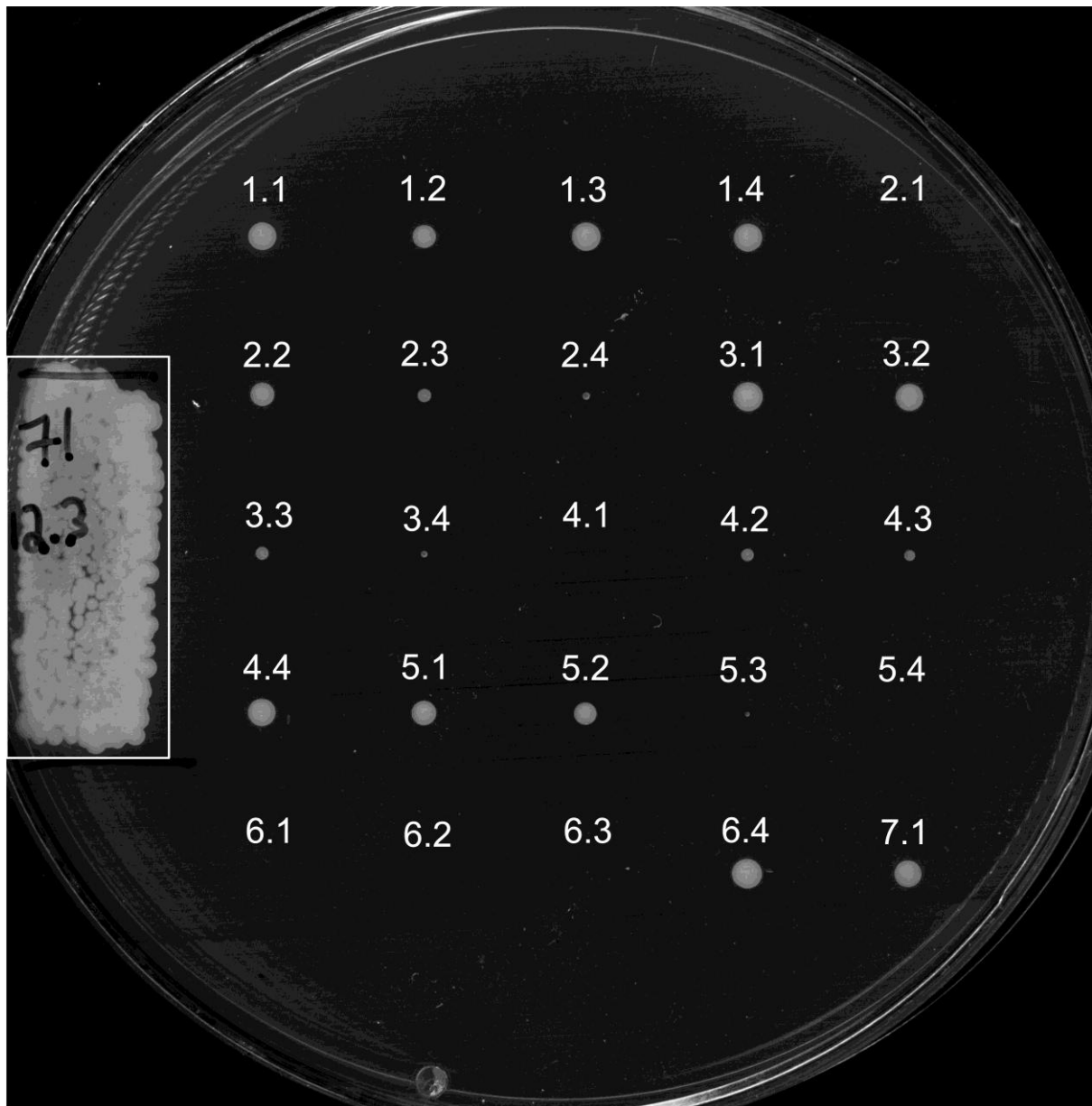
Figure 1 Variation in spore colony size. Photograph showing 25 spores from the same strain (Plön4) placed on an agar plate in a 5 x 5 grid, and incubated 25°C for 3 days. The location of each spore is labeled with a number. The number before each decimal point indicates the tetrad each spore came from, i.e. spores with the same first number are from the same tetrad. Spores were taken from 6 complete tetrads, plus a single spore from a seventh

tetrad (7.1). The rectangle indicates the area where a sample of digested spores was deposited.
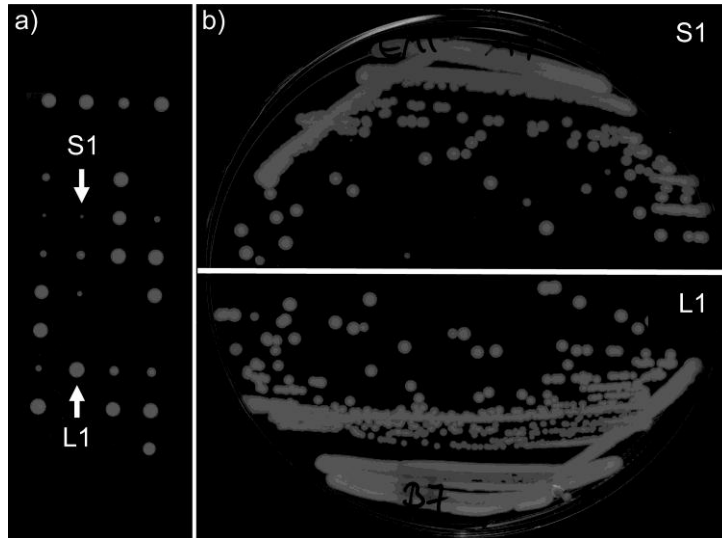
Figure 2 Comparing sizes of colonies produced from isogenic spores versus secondary colonies produced by their streak. Panel a) shows an example of a pair of small (S1) and large (L1) 'spore colonies', panel b) shows 'vegetative colonies' derived from the spore colonies by streaking.
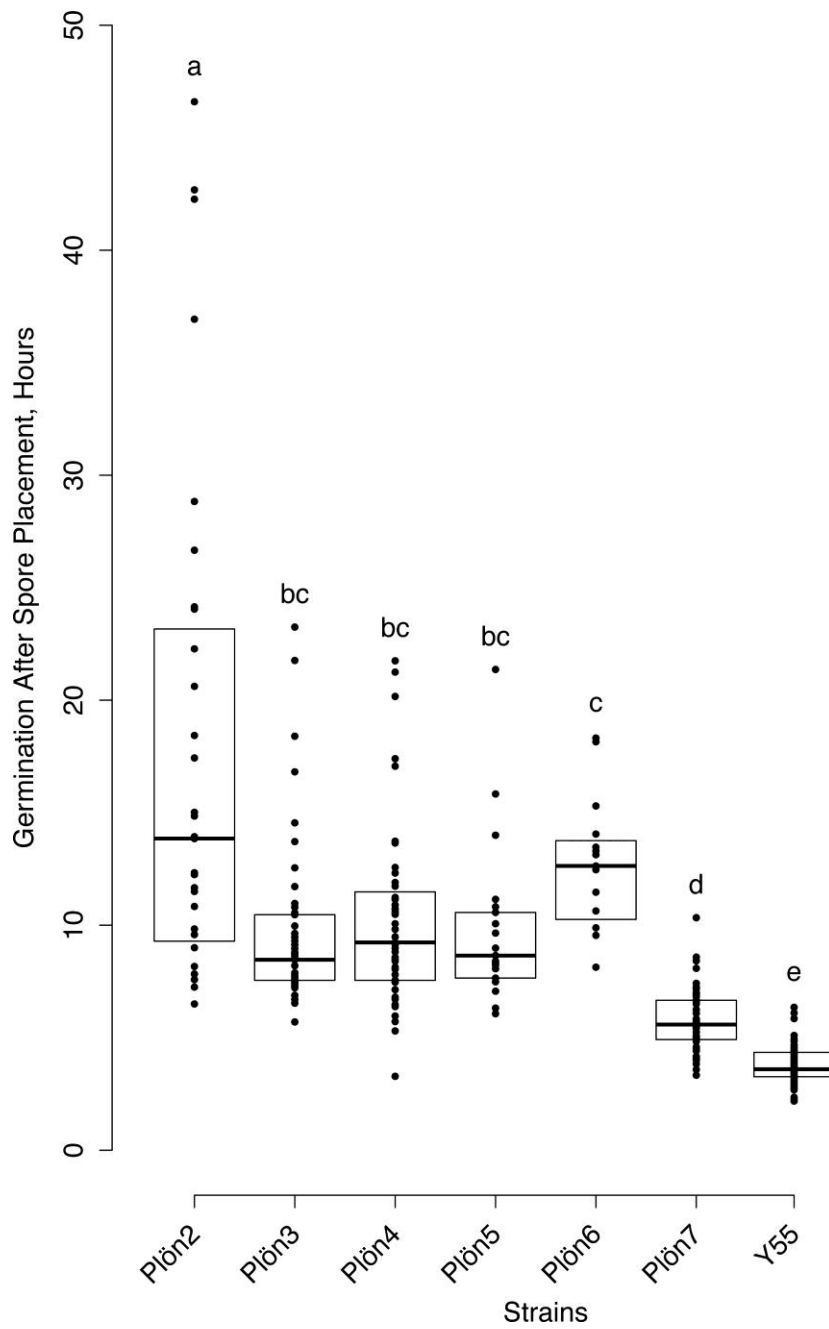
Figure 3 Variation in germination timing. Box plots show the mean and the first and third

quartiles of spore germination time within each strain, as determined by time lapse

microscopy over the course of 48h. Every data point represents a single spore. 80 spores

(from 20 tetrads) from each strain were tested, but only those that germinated are represented

here (see Table 2). Letters signify significant pairwise differences in variance between strains

(Tukey test – see Results).

Colony Radius (mm)

Days on Sporulation Medium
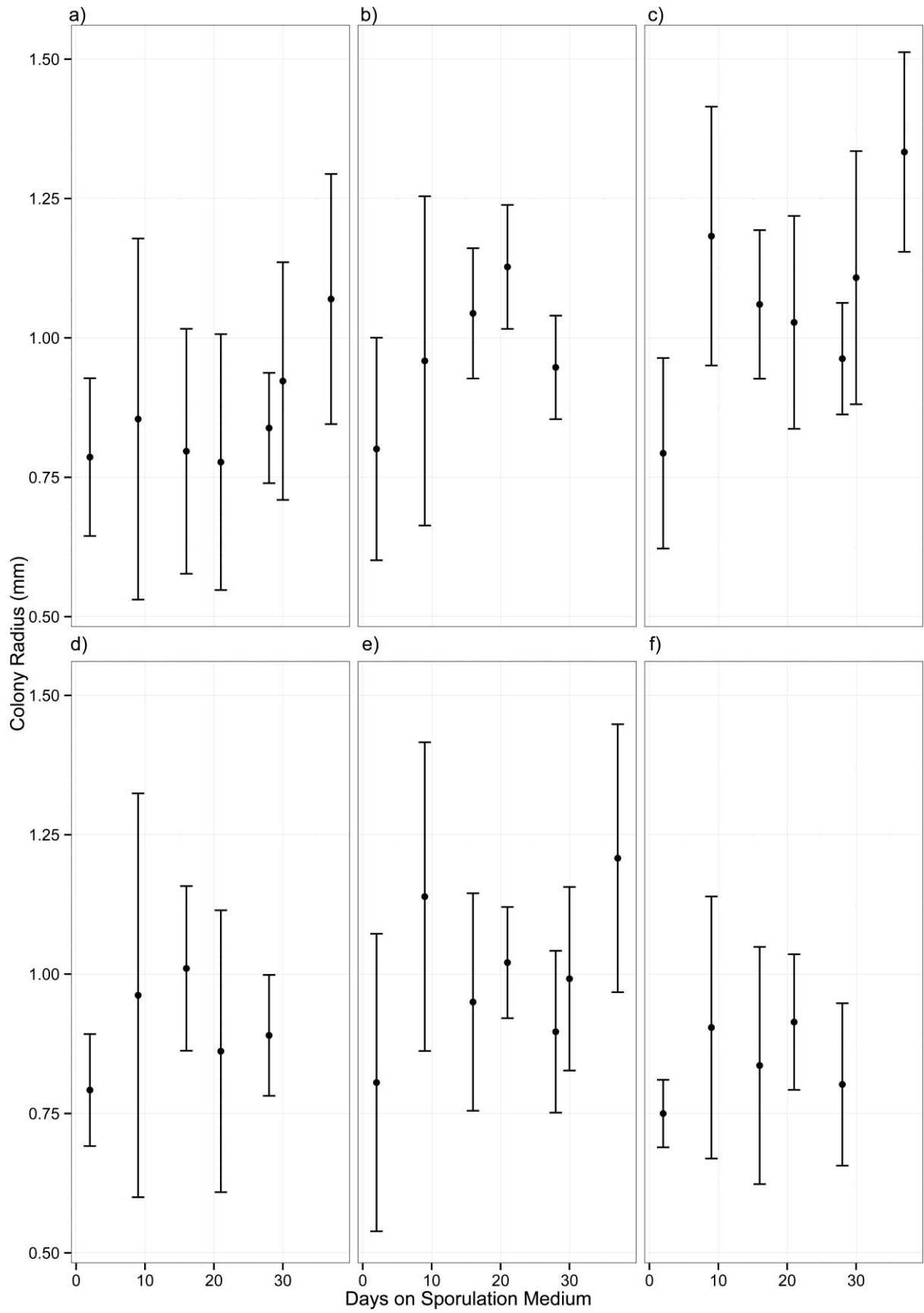
Figure 4 Variation in colony size as a function of spore age. Mean (round points) and standard deviation (error bars) of germinated spore colony size as a function of spore age (i.e. days on sporulation medium before testing individual spore germination and measuring the resulting spore colonies). The six plots, a) through f) show the results for the six different wild strains, Plön2 through Plön7. 50 spores per strain were tested at each time point, except for the 9 day time point when 100 spores per strain were tested. Only visible colonies were measured.
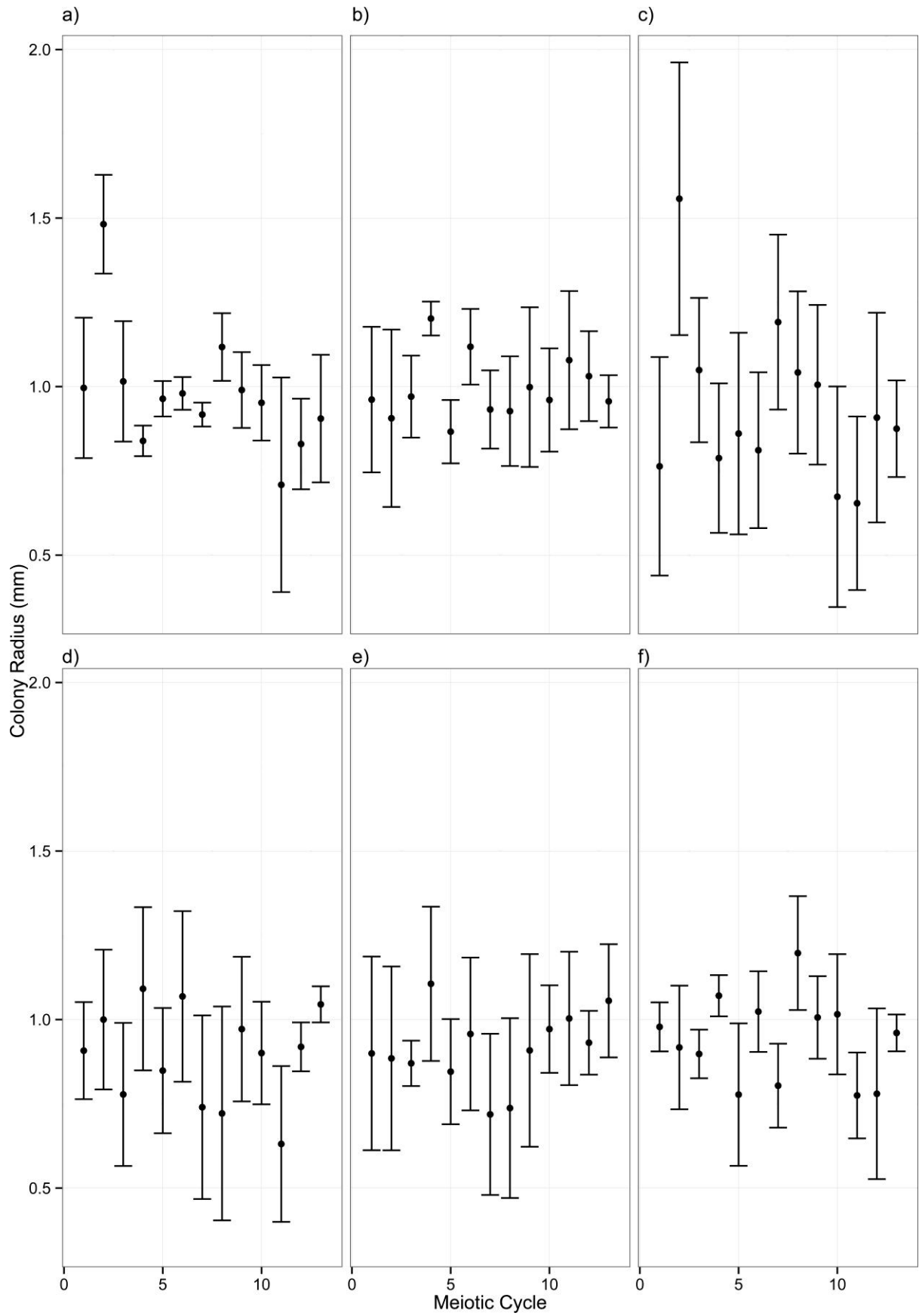
Figure 5 Variation in colony size as a function of the number of preceding meiotic cycles. Mean (round points) and standard deviation (error bars) of colony size as a function of the number of preceding meiotic cycles (see Methods). Plots a) through f) are strains Plön2 through Plön7. 50 spores per strain were tested after every meiosis. Only visible colonies were measured.
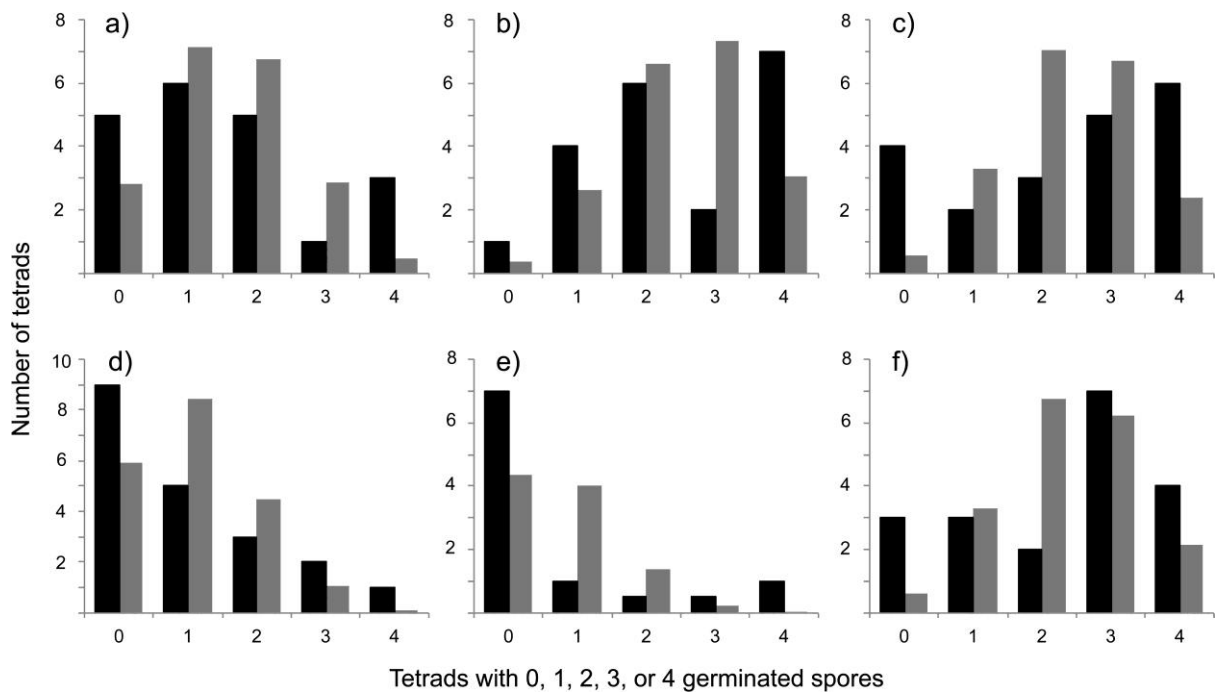
Figure 6 Distribution of germinated spores among tetrads. Number of tetrads with 0, 1, 2, 3, and 4 germinated spores after 48 hours, as determined by time-lapse microcopy. Black bars show the observed data, grey bars show the expected number of tetrads using a Poisson distribution predicted from the overall germination rate (see Results). The six plots, a) through f) show the results for the six different wild strains, Plön2 through Plön7. 20 tetrads (80 spores) arrayed on a single plate were tested, for every strain.

Table 1 Variation in the size of colonies produced by isogenic spores

Table shows the means and ranges of colony sizes (colony radius in mm) for visible colonies produced by isogenic spores of six wild *S. paradoxus* strains (Plön2-7) after three days of incubation, and proportions of germinated spores (i.e. those that produced visible colonies) after three days and 14 days, respectively. Numbers in brackets show the number of spores that germinated. n = total number of spores tested per strain.

strain	n	mean visible colony size (mm ± SD)	range in visible colony size (mm)	proportion of germinated spores	
				after 3 days	after 14 days
Plön2	250	0.93 ± 0.24	0.21 - 1.25	0.58 (145)	0.772 (193)
Plön3	250	0.99 ± 0.23	0.21 - 1.39	0.812 (203)	0.9 (225)
Plön4	250	0.87 ± 0.25	0.18 - 1.28	0.74 (185)	0.868 (217)
Plön5	250	0.90 ± 0.16	0.12 - 1.16	0.776 (194)	0.812 (203)
Plön6	250	1.00 ± 0.20	0.23 - 1.18	0.608 (152)	0.772 (193)
Plön7	250	0.89 ± 0.15	0.21 - 1.18	0.82 (205)	0.876 (219)

Table 2 Variation in germination timing of isogenic spores

Table shows means and ranges of germination time (in minutes) of spores that germinated within 24 hours, for six wild *S. paradoxus* strains (Plön2-7) and a laboratory *S. cerevisiae* strain (Y55). n = total number of spores used per strain. Also shown are the proportions of spores that germinated (and whose germination time was therefore measured) after 48h in this experiment. Numbers in brackets indicate the total number of germinated spores per strain. Chi-square tests compare the distribution of germinated spores within tetrads and within perpendicular quadruplets (as described in Methods and Results) with the expected Poisson distribution predicted from the overall germination rate. Significant p-values are in bold (behind slash).

strain	n	mean germination time (min ± SE)	range in germination timing (min)	proportion germinated after 48 hours	χ^2 statistic (p-value) for spores in tetrads	spores in perpendicular quadruplets
Plön2	80	1059.2 ± 123.3	390-2796	0.39 (31)	17.94 / **0.001**	3.99 / 0.41
Plön3	80	582.8 ± 31.3	342-1395	0.63 (50)	10.66 / **0.03**	3.18 / 0.53
Plön4	80	609.4 ± 35	197-1305	0.59 (47)	28.96 / <**0.001**	0.96 / 0.92
Plön5	80	586.1 ± 46.5	364-1282	0.26 (21)	12.94 / **0.011**	0.61 / 0.96
Plön6	80	760.1 ± 46.4	488-1099	0.19 (15)	167.49 / <**0.001**	2.85 / 0.58
Plön7	80	346.9 ± 12.6	200-620	0.58 (47)	14.79 / **0.005**	0.82 / 0.94
Y55	80	227.3 ± 5.6	131-381	0.95 (76)	8002.16 / <**0.001**	0.51 / 0.97