Sexual Selection in Yeast

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Declaration

I, Carl Alessandro Moreno Smith confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Saccharomyces yeasts are unique as a model system in evolutionary biology. They offer all the traditional benefits of fast generation times and easy maintenance found in other microbes such as *Escherichia coli*. In addition, *Saccharomyces* are diploid eukaryotes capable of asexual and sexual reproduction. In this thesis I develop *Saccharomyces* as a model organism for the study of sexual selection. I show that its mating pheromone is costly to produce and maintain, and that this cost is greater for lower quality individuals. This suggests that the pheromone may have evolved as a sexual signal under the Handicap Principle. I show that size can offer direct benefits during mating and that these are in fact selected for. I show that preferential mating also takes place to help clear deleterious mutations from a population. I also investigate mating barriers in yeast to better understand how yeast mating may take place in nature.

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1. General Introduction

1.1 Overview

This thesis details work developing the yeast *Saccharomyces cerevisiae* as a model organism for the study of sexual selection. *S. cerevisiae* has several benefits over more traditional model systems used in the study of sexual selection (such as birds and fish). It has a short generation time and easy maintenance characteristic of other microbial model systems such as *Escherichia coli* and *Chlamydomonas reinhardtii*, which make it better suited for evolutionary experiments. But unlike *E. coli*, *Saccharomyces* yeasts are eukaryotes capable of both sexual and asexual reproduction. Yeast has an easily manipulated sexual system, and as a result of being used in molecular and genetic research for decades, it has an unrivalled understanding and a library of molecular techniques as well as a fully sequenced genome for several species. However, yeast falls short as a model system due to the lack of knowledge surrounding its natural ecology. Without this information it is difficult to fit context to results and interpret them within a natural evolutionary system.

In this thesis, the *Saccharomyces cerevisiae* mating pheromone is first characterised as having the properties of an honest sexual signal evolving under the handicap principle. The possible reasons for sex in yeast are then investigated by testing for both short-term and long-term benefits of sexual reproduction. These studies attempt to help understand the poorly explored natural mating behaviour of yeast and continue to develop *S. cerevisiae* as a powerful tool in the field of evolutionary biology.

1.2 Evolution of sex

1.2.1 Modes of reproduction

Asexual reproduction takes place through either vegetative propagation or parthenogenesis. Vegetative propagation is observed in plants. New individuals arise from a group of cells such as a stem, rather than from a seed (Leaky 1985). Where parthenogenesis is present (as in yeast (Herskowitz 1988), progeny are formed from a single mother cell or unfertilised egg (as in several vertebrates (reviewed by Neaves & Baumann 2011)). Asexual organisms produce offspring that are identical to themselves (clones) with the exception of any new mutations that may have occurred during replication.

Sexual reproduction usually refers to the formation of and union of two genomes by the coming together of gametes. These gametes may come from separate individuals (out-crossing) or from the same individual (self-fertilisation). One of the key characteristic processes associated with sexual reproduction is the independent segregation of chromosomes and the recombination among loci. This creates genetic variation between gametes. Many species have distinguishable sexes that produce gametes of different sizes. Where gametes are of a similar size, individuals are not of different sexes, but rather of different mating types. When an individual is capable of producing both types of gametes, it is said to be hermaphroditic. In some cases hermaphrodites are capable of self-fertilisation as in several plant species (Lande & Schemske 1985). However, some hermaphrodites simply change sex following

environmental cues. This is most commonly seen in fish (Devilin & Nagahama 2002).

1.2.2 Asexual reproduction versus sexual reproduction

Maynard Smith asked the question "what use is sex" in 1971. Previous to this question, sex was accepted as a process that promoted greater genetic variation in offspring. This greater variation would in theory lead to better adaptation. The problems with sex were not given much consideration. But upon some consideration, sex seems a terrible strategy for reproduction. An individual who has survived through to reproductive age will have a genome that is sufficiently well adapted to their current environment. So why should it want to break up this successful combinations of genes by having sex? In 1975, Williams argued that asexual organisms will produce offspring that will stay close to the parent, so will have a genome that is well suited to their environment. In addition to this, he also states that natural selection will be more intense on sexually produced offspring, due to their longer vulnerable dormancy period. The act of sex itself can also be costly. Individuals need to find a mate. This takes time and energy. Plants offer a good example of this cost, as they need to expend resources on floral displays and nectar to ensure pollination. In some cases securing a mate can be very dangerous where physical competition between males takes place (arachnids: Austad 1983, cervids: CLutton-Brock 1982, insects: Hamilton 1979, anurans: Wells 1977).

Having secured a mate, the act of mating itself can also be costly. During mating individuals are left exposed and vulnerable to predators. In some cases such as with *Drosophila*, the seminal fluid is harmful to females and actually reduces their fitness (Chapman et al 1995). There are also several sexually transmitted diseases that make sex a potentially hazardous undertaking.

These are mostly short-term problems that a sexual individual will have to face. But sex is also a problem at a population level. In an asexual population, one individual can produce offspring. In a sexual population, only a breeding couple may produce offspring. So if both breeding couples and asexual individuals produce the same number of surviving offspring, an asexual population will produce twice as many offspring (Fig. 1.). However, it is important to note that in cases where both parents in a sexual species help care for the offspring, the number of surviving offspring is higher and so helps counterbalance this particular cost of sex.

With such disadvantages associated with sexual reproduction, it is difficult to see why it evolved. But there must be a significant long-term advantage associated with it. After all, the majority of multicellular eukaryotes use some form of sexual reproduction (Williams 1975, Maynard Smith 1978, Burt 2000). Many asexual eurkaryotic lineages retain structures associated with sexual reproduction (as with the dandelion *Taraxacum officinale* that retains non-functional stamens and brightly coloured petals). If a parthenogenetic lineage had persisted for several millions of years, then it should have diverged enough to form a morphologically distinct form from its sexual relatives. However, such

divergent morphology only exists in very few eukaryotes such as bdelloid rotifers (Normark et al 2003). Phylogenetic studies such as those done on snails (Neiman et al 2005) have shown that asexual lineages are often descended from older sexual progenitors. This suggests that not only have most asexual species emerged relatively recently, but also the lack of ancient asexual forms may be indicative of asexual organisms being more susceptible to extinction. This is thought to be due to the irreversible accumulation of deleterious mutations in asexual systems (Muller 1964, Lynch & Gabriel 1990), while the lack of variation prevents evolutionary responses to selection pressures (Maynard Smith 1986, Rice 1983, Hamilton et al 1990, Barton & Charlesworth 1998, Burt 2000). It seems then that sexual reproduction has a long-term advantage over asexual reproduction, but for it to even get to that stage there must be a short-term advantage to establish it. Organisms such as *Saccharomyces cerevisiae* that reproduce both sexually and asexually, may help understand this problem. They reproduce asexually the majority of the time, and only have sex in response to a change in their environment that causes meiosis and the formation of spores (Croes 1967). This suggests that sexual reproduction may be a response to special situations (Williams 1975).

1.2.3 Possible short-term advantages of sex

There are many hypotheses on why sex and recombination have such a high prevalence, but the majority are variations on the following themes.

1.2.4 Repair of damaged DNA

Repair of breaks in a DNA molecule can take place by copying the sequence from a homologous chromosome during recombination (Bernstein & Bernstein 1991). Under this hypothesis, any new combinations of alleles resulting from sexual reproduction are simply side effects of the DNA repair. This theory offers a worthwhile benefit to sexual reproduction, but it does not offer an explanation for some of the more elaborate mechanisms involved in sex. So it is far more likely that the maintenance of sex has far more to do with variation and selection (Maynard Smith 1988, Barton & Charlesworth 1998).

1.2.5 Variability of relatives

Sex and recombination cause differences between the genotypes of parents and each offspring. This variation may help prevent the spread of pathogens through reproduction. Pathogens can be specialised to the individual host genotype (Day 1974, Johnson & Taylor 1976, Klein 1979). So by producing offspring with a different genotype, it can help prevent transmission of these specialised pathogens (Rice 1983).

Sibling offspring will have different genotypes as well. This has two possible implications. Where selection pressures are strong (Barton & Post 1986, Bulmer 1980, Taylor 1979), competition between siblings will make sure that the best genotype will survive for those particular conditions (Williams 1975, Williams & Mitton 1973). However, if selection pressures remain constant, the advantage of sex diminishes, as

an asexual population will continuously produce offspring that are perfectly adapted to their environment.

An alternative view on sibling competition suggests that genotypic variation actually reduces the intensity of competition (Bell 1982, Maynard Smith 1978, Price & Waser 1982). An asexual species will produce genetically identical offspring which all compete for the same resources within their environment. However, a sexual species will produce genetically distinct offspring, which may allow them to use different resources in the same environment. This would mean that the same environment could potentially maintain a larger sexual population relative to an asexual population. However this theory was criticised as being somewhat incomplete (Wilson & Gleeson 1983). If multiple niches exist in an environment, an asexual population should invade them eventually through mutation. Once there, it would outcompete a sexual population due to the two fold cost of sex described earlier (Fig. 1.). For the sexual species to outcompete an asexual species, many of these niches would have to fluctuate in their existence. As a niche disappears, then so would asexual species within it. However, many of the alleles that are adapted to that niche can still survive in sexual species until it reappears once more.

1.2.6 Preventing mutational deterioration

In 1964 Muller stated that in asexual lineages, the loss of a mutation free genotype is irreversible with the exception of backward mutations. This is more commonly known now as 'Muller's ratchet'. This

irreversible process leads to a steady decline in fitness and population size that may eventually lead to extinction (Lynch et al 1993). However, in a sexual population, mutations can be eliminated through recombination. This hypothesis may help explain why asexual populations are more susceptible to extinction, but in the short-term it may not be enough to offset the previously stated disadvantages of sex. Unless the population size is extremely small (Lynch et al 1993), it would still take about 100 generations for asexual species to become maladapted to their environment. Different severities of mutation will also affect this process. If a mutation is particularly deleterious, the best class of genotype will be much more prevalent, making the process very slow. However, if mutations are only slightly deleterious, then many more will have to appear before causing maladaption.

1.2.7 The mutational deterministic process

If deleterious mutations at different loci reduce fitness more when they are combined in the same genotype than would be expected from their separate effects, they are said to show 'synergistic epistasis'. Such a scenario would allow sexual reproduction to eliminate these mutations very quickly (Kondrashov 1988). This is because recombination in a sexual population will bring these mutations together much faster than in an asexual population. So the mean fitness of a sexual population will ultimately be higher than that of an asexual population. This model relies on a high frequency of new deleterious mutations (one per genome per generation) as well as synergistic epistasis between them to counteract

the high cost of sex. Unfortunately the epistatic effect required may not always be evident. This was shown to be the case with deleterious mutations in *Escherichia coli* (Elena & Lenski 1997). While more recent studies (MacCarthy & Bergman 2007) show that coevolution of epistasis and recombination may in fact favour asexual reproduction.

1.2.8 Adaptation to new environments

Perhaps the most obvious advantage of sex is its ability to speed up the rate of adaptation. In an asexual population, beneficial alleles *A* and *B* would only be combined when one mutation occurs in a lineage that already has the other beneficial mutation. However, in a sexual population, both beneficial alleles can occur independently in different lineages and then come together through recombination. So you would see a faster fitness increase in the sexual population. This will only be the case in large populations though. In a large population you are far more likely to find different favourable mutations present at any one time. However, if the population size is small, the interval between favourable mutations appearing is such that sexual populations do not adapt faster (Crow & Kimura 1965) (Fig.2.). This theory was also backed up with experimental data using the model system *Chlamydomonas reinhardtii* (Colegrave 2002), to show that adaptation is faster in large sexual populations, but that the effect is lessened significantly in small populations.

1.2.9 Adaptation to fluctuating environments

Due to the mixing of alleles that takes place during sex, sexual organisms are able to produce variation amongst their progeny. Should the environmental conditions suddenly change selection pressures, a sexual organism will be able to produce offspring with a new, better adapted, combination of alleles far quicker than an asexual organism. For this advantage to remain realistic, the environment will have to fluctuate quite often to maintain variation and avoid fixation. (Maynard Smith 1980).

The Red Queen hypothesis offers a scenario where this may be taking place (van Valen 1973). Here continuous adaptation is required to maintain relative fitness between co-evolving systems. This is popularly characterised by parasite/host interactions. The host is under constant pressure to find new combinations of alleles to resist the parasite. At the same time the parasite is under similar pressure to evolve to be invade the host. Each species must continually evolve to keep up with the evolutionary changes of its competitors.

1.3 Sexual selection

1.3.1 A short history

Although it is difficult to find a single unifying theory for the prevalence of sex, it does remain clear that it has the potential to offer many fitness advantages be they DNA repair, or potential for better adaptation and ultimately avoid extinction. Such an important process

therefore needs rules to operate by to make sure that the cost is worthwhile. This relies on bringing the best genotypes together to create the best possible combination of alleles, resulting in competition for mates.

Competition for mates is key to Darwin's concept of sexual selection (Darwin 1859, 1871). Darwin was puzzled by the often-elaborate traits found in males and the reason for their existence. These traits do not give the individual an advantage in survival as outlined by his theory of natural selection (Darwin 1859), but rather were "perfected through sexual selection, that is, by the advantage acquired by certain males over their rivals" (Darwin 1871 p.257). While natural selection is usually caused by environmental factors, sexual selection is driven by sexual rivals and mates (Ghiselin 1974). Despite Darwin proposing sexual selection as an evolutionary force in the 19th century (1859, 1871), it would only be a century later that it became a more popular field of study with the publication of several books on the topic (Ghiselin 1974, Williams 1975, Maynard Smith 1978).

1.3.2 Different methods of mate selection

Specific systems have evolved to select the best possible mate. Most sexual systems have two dimorphic sexes (typically male and female). In these, the female usually invests more in each mating than the male does. Their investment includes larger and fewer gametes, and can also often include raising the offspring as well (Bateman 1948, Trivers

1972, Clutton-Brock 1991). Ultimately though, the number of offspring produced by males is the same as that produced by females if the sex ratio is equal (Fisher 1930). So why should one sex provide less care for their offspring? One possible reason is the uncertainty of paternity. Trivers (1972) argued that in promiscuous societies or where a female's brood is sired by multiple males, each male has a lower expected relatedness to the offspring, making females more likely to provide care. However this argument was countered by noting that, a male that does not provide care does not have a higher paternity probability in any future matings (Maynard Smith 1978, Grafen 1980, Werren et al 1980, Westneat & Sherman 1993). However there is still some support of the theory that paternity does matter (Trivers 1985, Queller 1997).

Males may also invest less in the care of offspring due to their greater potential reproduction, so they stand to lose more than females by concentrating on fewer offspring (Maynard Smith 1977, Clutton-Brock 1991). Because of sexual selection, variance in male reproductive success is higher than that of females (Bateman 1948). So while males and females may produce the same number of offspring, fewer males than females are mating. Male parental care will be more costly, as he stands to lose more than a female by focusing on producing less offspring. Therefore the cost associated with parental care will be stronger in males than in females as a result of sexual selection.

Sexual selection can also arise in monogamous societies as well if the sex ratio is skewed, or if there is a difference in quality of available mates (Darwin 1871, Fisher 1958). Where sex ratio is skewed, not all

males will be able to mate, resulting in competition similar to that found in polygamous populations. However, if sex ratio is equal, a difference in quality of mate will produce competition. In some birds, females in the best condition are ready to mate earlier in the season, leaving them more time to care for their young (Price et al 1988). The first males to mate will then get better care for their offspring. Where parental care is equal, it is still important to choose a good quality mate, as they are likely to survive longer and be better able to provide resources or defend against predators. So traits that improve mating ability should be preferred in monogamous species (O'Donald 1987, Price et al 1988, Kirkpatrick et al 1990).

Due to the difference in gametes and the tendency for maternal care, mate competition usually takes place in males, while mate choice should usually take place in females (Reviewed by Andersson 1994). However, in the few cases such as with some species of pipefishes and seahorses (Fiedler 1954, Berglund et al 1986) where paternal care is more common, there can be a reversal in sexual dimorphism and sex roles. Sometimes, the two sexes may be monomorphic and provide similar input to each mating. This is the case with the yeast *Saccharomyces cerevisiae* and its two mating types *MATa* and *MATα*. In species such as this, sexual selection may still arise, but the lack of dimorphism may be due to similar pressures on both sexes (or mating types) (Darwin 1971, West-Eberhard 1983, Trail 1990), as would be the case with *S. cerevisiae* (Payne 1984, Herskowitz 1988).

There are two ways in which sexual selection acts, mate competition (intra-sexual selection) and mate choice (inter-sexual selection). With intra-sexual selection, males (usually) compete with each other for access to females or territories where females go (male bullfrogs (Howard 1978), damselflies (Marden & Waage 1990). Competition between males takes many forms, the most spectacular being fighting directly with each other (red deer stags (Lincoln et al 1970, Appleby 1980). But competition can also be much more cryptic as with sperm competition where males hamper each other's chances of fertilising females. Male *Orthetrum cancellatum* have a barbed whip at the end of their penis to scrape out any sperm from previous copulations with other males (Waage 1979). Male *Moniliformes dubius* cement the female's genital opening after mating preventing fertilisation by other males (Abele & Gilchrist 1977).

With inter-sexual selection males do not compete directly with each other in physical contests. Rather they exhibit traits that make them more attractive to female. The female then chooses the male with the most impressive display. The most recognisable examples of this are in birds. Males of several bird species develop elaborate or brightly coloured plumage (birds of paradise (Cooper & Forshaw 1977, swallows (Møller & de Lope 1994)) to attract females. Sometimes it is a craft that attracts females, as is the case with male bowerbirds (Borgia et al 1985, Diamond 1988) that use their building skills to attract potential mates.

1.3.3 Benefits of mate selection

For such elaborate and costly courtships to exist there must be a benefit associated with them. When females chose mates on the basis of material resources on offer, the benefit is not necessarily genetic. Instead the resources offered by the male can help increase the likelihood that her offspring will survive. This can be seen in North American Bullfrogs (*Rana catesbeiana*). Males wrestle and call to control territories in ponds where females lay their eggs. Some of these territories offer much better environments for fertilised eggs. The eggs can come under attack by leeches (*Macrobdella decora*). However, if the water is warm, the eggs develop faster (so they are under threat for less time), and if the vegetation in the water is not too dense, the eggs can clump together into defensive ball formations (Howard 1978). In the common tern species (*Sterna fuscata*), males offer a similar non-genetic benefit. During courtship, males bring food to the female. Their ability to bring food during courtship has been shown to correlate to their ability to help feed resulting chicks. So here the female selects a partner based on how well he will help feed her offspring (Nisbet 1977).

Sometimes, males are chosen on the basis of elaborate traits such as striking plumage in birds (birds of paradise (Cooper & Forshaw 1977, swallows (Møller & de Lope 1994)). These do not offer any direct benefits to the female such as material resources. In many cases it would seem that these exaggerated traits could even hamper the male (Møller & de Lope 1994), making them poor flyers, or making them more visible to predators. It is difficult to see why such traits would have evolved and why females would find them attractive.

1.3.4 Fisher's runaway process

In 1930 R. A. Fisher put forward that these elaborate displays simply came about because they are, in fact, attractive to females. He argued that originally the trait might have been associated with a particular benefit. For example, slightly longer tail feathers may have actually improved a male birds ability to fly thus allowing them to gather more food and avoid predators better. Since Fisher, alternate starting points have been suggested. There may have been an existing sensory bias (Ryan et al 1990), or it may have been as basic as the trait making a mate easier to detect (Arak 1983). As long as there was a genetic basis for longer male tail feathers, the trait will be passed on to their sons. At this stage if a gene causes females to prefer males with longer tail feathers, it will begin to spread throughout the population as a result of linkage disequilibrium with the gene for longer tail feathers. Females that prefer males with longer tail male feathers will not only produce sons that can fly better, but are also more attractive to females. As both genes spread through the population, both sexes gain. Males with longer tail feathers can fly better and are more attractive. Females produce sons that fly better and are more attractive. However, the resulting positive feedback loop drives male tail length past its optimum length. Once decreased survival counterbalances attractiveness to females, the male tail feathers will stop increasing in length. The result is an exaggerated trait that may hamper the male, but is still attractive to the female.

Fisher's theory of runaway selection (1930), helps explain why exaggerated sexual traits have evolved. But there are some important points to consider. The cost of female is crucial to the outcome of a trait evolving under the Fisherian process. If female choice is costly then exaggerated male traits cannot be sustained at equilibrium (Kirkpatrick 1985, Pomiankowski 1987, Pomiankowski et al 1991). There are several examples of cost of female choice, such as reduction in fertility (Engelhard et al 1989) and increased risk of predation (Frederick 1987), so it should not be ignored.

For Fisherian runaway evolution to work, biased mutation on the male trait has to be such that there is an excess of poorly ornamented males in the population. Being poorly ornamented makes them less attractive mates, so the cost of female mate choice is then counterbalanced by the traditional benefit of mating with an attractive mate to produce more attractive sons (Pomiankowski et al 1991).

Testing Fisher's runaway process experimentally poses a few problems. One would first need to show that there is genetic variation for both the ornament and the preference for it and that covariation is present between these genes. Fisher assumes that the only benefit of female mate choice is increased mating success for her sons. So one would also need to show that the ornament does not represent any other benefits such as those outlined by the handicap principle (Zahavi 1975 & 1977) below. Testing for a correlation between extreme ornaments and increased viability presents difficulties. To test Fisher's process one would need a negative result from such a test. Such a result is difficult to

trust, given the many factors that may influence it (such as sample size and other variables).

Studies do exists in this area however. Genetic correlation between male display and female preference has been shown in guppies (Houde 1988, Houde & Endler 1990). The stream systems in Trinidad are home to many guppy populations. Males develop bright orange or blue spots on their bodies that induce courtship by females. Males from different populations vary in how they are marked. Those from streams with no predators form much larger spots than those from populations under predation by other fish. Females from predator free streams are shown to have a stronger preference for males with large spots than do females from streams exposed to predators (Houde 1988). This preference also persists after several generations of laboratory rearing, showing a genetic correlation between male trait, and female preference for that trait. It is still however very difficult to show that the size of spot does not indicate male viability as outlined by the handicap hypothesis (Zahavi 1975, 1977). Indeed the colour of the spot is likely to be influenced by environmental factors (Kodric-Brown 1989, Houde & Torio 1992).

1.3.5 The handicap hypothesis

Amotz Zahavi proposed a different hypothesis for the existence of exaggerated sexual ornaments (1975, 1977), where mate preference is favoured by selection if the ornament handicaps survival. There are three interpretations of this.

The first interpretation is based on epistatic fitness interactions between the ornaments and general viability (Maynard Smith 1976, Davis and O'Donald 1976, Bell 1978). All males can produce a small ornament and not suffer for it. A large ornament may bear a significant cost though. Here only males with a high viability can maintain it. So after males with lower viability have died as a result of the cost of the ornament, surviving males will generally be of a higher viability. So females that mate with these males will in turn produce offspring of a better viability. This interpretation is now somewhat redundant, as Zahavi stated that the handicaps should have a condition-dependent nature, such that ornament size and condition is directly influenced by the bearer's viability. Iwasa et al proved this to be true in 1991, as they showed through mathematical modelling that epistatic fitness interactions alone were not enough to cause stable evolution of costly female mate preference.

The conditional handicap interpretation however suggests that the ornament phenotype increases in correlation with the bearer's condition, accurately reflecting genetic quality and viability (Zahavi 1977, West-Eberhard 1979, Nur & Hasson 1984, Kodric-Brown & Brown 1984, Andersson 1986, Zeh & Zeh 1988). Some experimental data supports this interpretation is being plausible in swallows (Møller 1988 & 1989), wolf spiders (Kotiaho 2000) and even in microbes such as budding yeast (Smith & Greig 2010). Swallows perhaps are the more classic example. Male swallows with artificially elongated tail feathers were shown to be preferred as mates by mating quicker and more often (Møller 1988). However, males with artificially elongated tail feathers were shown to

bear a greater cost than males with inherited elongated tail feathers (Møller 1989). In subsequent moults the natural tail length of males with artificial tail lengths was actually seen to decrease, while those with inherited long tail feather length remained constant. This is example strongly supports the interpretation of the handicap hypothesis as acting through condition-dependent traits.

Thirdly, the handicap hypothesis has also been interpreted as a revealing handicap (Hamilton & Zuk 1982). Here the construction of the male ornament is not affected by general viability. But its maintenance is subject to the general condition of the male. Originally it was suggested that such a system would be an accurate indication of an individual's parasitic load. But it may also apply if general upkeep of ornaments such as elaborate feathers is more difficult for less viable males (Iwasa et al 1991).

Fisherian evolution of female choice for exaggerated male traits and the handicap theory should not be viewed as mutually exclusive. While there are studies that lend evidence to both theories, neither has been disproven satisfactorily. They are not incompatible with each other (Iwasa et al 1991), so future studies may do well to consider both to give a fuller interpretation of their results.

1.4 Using microbes as model organisms

Our understanding of the processes by which sex and sexual selection may have evolved has come from mathematical models that test theoretical principles. These theories help give an evolutionary context to

what we observe in nature. But there have been many important experimental tests to show some of the benefits of sexual reproduction. *Drosophila* have been used to great effect to show the importance of recombination to fitness and level of adaptation (Rice 1994, Bachtrog & Charlesworth 2002). The obstacle to testing evolutionary theory is the slow nature of evolution. Observations from the wild, and even experiments using model organisms such as *Drosophila* can only offer a small snap-shot of the evolutionary process. An ideal experiment would show evolution in all its stages, from mutational variation, through to adaptation of the population. Unfortunately to do this in most model systems would take a staggering amount of time.

Microbes are now often used as model organisms for testing evolution. Their short generation times allow evolution to take place under laboratory conditions. Perhaps the best example of this is the long running experiment using *Escherichia coli* at Richard Lenski's laboratory. They created 12 identical starting populations from a single bacterial colony. These were maintained for over 46,000 generations through daily serial propagation into fresh media. At regular intervals, samples of each population are taken and frozen to form a virtual fossil record. When needed, these can then be revived to compare evolved strains directly with their ancestors. This long running experiment has shown that adaptation was much faster at the beginning of the experiment, occurring at similar rates across the 12 replicates as their morphology changed and their fitness improved. However, the 12 lines eventually began to reach different fitness peaks (Lenski & Travisano 1994). These different peaks

are remarkable given that each of the 12 lines started off as identical clones, and their environment remained constant throughout. The differing fitness peaks observed between the twelve lines represent independent beneficial genotypes occurring and sweeping through the population to fixation. This shows the important roles of chance events such as random genetic drift and random mutations (Travisano et al 1995), in giving rise to differences between previously identical populations subjected to the same selection pressures.

Simple yet powerful experiments such as these have utilised microbes to contribute a vast amount to our knowledge of the evolutionary process (Travisano et al 1995, Elena et al 1996, Sniegowski et al 1997, reviewed by Elena & Lenski 2003). Although using bacteria for experimental evolution has advanced our understanding, and will probably continue to do so, bacteria do have one key draw back. Bacteria are haploid, asexual prokaryotes. Most evolutionary theory, and more importantly, sexual theory is applicable only to diploid sexual eukaryotes.

1.5 *Saccharomyces* as a model organism for sexual selection

The budding yeast *Saccharomyces cerevisiae* has been increasingly used as a model system to study evolution (reviewed by Zeyl 2006). This yeast has all the benefits associated with bacteria such as *E. coli*. It has a very short generation time comparable to that of *E. coli*, it is very easy to maintain, it can be manipulated genetically with great ease, it has five closely related species which are similarly easy to manipulate and even has a fully sequenced genome (Zeyl 2000, Landry et al 2006). Of course

what makes *S. cerevisiae* an extremely useful system for the study of evolutionary processes is that it is a sexual eukaryote, often existing in diploid form. *Saccharomyces* yeasts have already been used to great effect in many studies (Zeyl 2006, Replanksy et al 2008) into the benefits and cost of sex (Zeyl & Bell 1997, Greig et al 1998, Goddard et al 2005), mutation rates (Zeyl et al 2001, Zeyl & DeVisser 2001) and speciation (Hunter et al 1996, Greig 2002, 2003, 2009, Delneri et al 2003, Dettman et al 2007).

S. cerevisiae has been used extensively as a model system in genetics and molecular biology. As a result there is a wealth of knowledge characterising it at a biochemical level as well as a multitude of useful molecular techniques. Unfortunately, despite the amazing level of detail to which *S. cerevisiae* is understood, there is remarkably little known about its natural ecology. Without this missing information, the system cannot be used to its fullest potential, as many of the results obtained cannot be explained in an evolutionary context.

1.6 The *Saccharomyces* life cycle

As with much of yeast, the *Saccharomyces* life cycle is very well understood (Herskowitz 1988) (Fig.3.). *S. cerevisiae* usually exists as a diploid of mating type *MATa/α* which reproduces asexually by budding daughter cells. When starved of nitrogen, these diploids will undergo meiosis resulting in four haploid spores held together inside an ascus. Two of these will be of mating type *MATa* and two will be of mating type *MATα*. When conditions support vegetative growth once more, spores of

opposite mating types can mate with each other or reproduce asexually as haploids. The mating type of a cell is controlled at a single locus (*MAT*). Depending on which allele is present, *MATa* or *αMATα*, cells will produce either a-pheromone or α-pheromone respectively. In addition to this, cell surface receptors are produced sensitive to the pheromone of the opposite mating type.

Pheromone production and the response to the pheromone during mating is well understood (Yu et al 2008, Kurjan 1992). The existence of the mating pheromones themselves were first determined by the ability of vegetatively grown *MATα* cells to induce morphological changes in *MATa* cells without physical contact. This suggested that *α* cells secreted at least one pheromone (Levi 1956). This was then further supported when cell culture supernatants from both *MATa* and *MATα* cells were seen to cause not only these same morphological changes, but induce cell-cycle arrest and agglutination in the opposite mating type (Duntze et al 1970, Bucking-Throm et al 1973, Shimoda & Yanagishima 1975, Hagiwa et al 1977, Betz et al 1978, Wilkinson & Pringle 1974).

The pheromone secreted by *MATα* cells was the first to be characterised (Bucking-Throm et al 1973, Shimoda & Yanagishima 1975, Hagiwa et al 1977, Betz et al 1978). It was found that the same 13-amino-acid peptide was responsible for the agglutination, cell-cycle arrest and morphological chances in *MATa* cells (Sakurai et al 1976, Stotzler et al 1976). A synthetic version of this peptide was also shown to induce the same changes in *MATa* cells, showing that it alone was sufficient to cause this response (Masui et al 1977, Ciejek et al 1977).

The pheromone is coded for by two genes *MFα1* and *MFα2* (Kurjan & Herskowitz 1982, Singh et al 1983), the resulting precursors are glycosylated and processed to produce mature *α*-factor (Fuller et al 1988, Kurjan 1991). These genes are semi-redundant, as a null mutation in either one does not cause any defects in pheromone production (except quantity (Jackson and Hartwell 1990) or mating ability (Kurjan 1985). However, loss of function to both genes causes sterility (Sprague and Thorner 1992).

The pheromone secreted by *MATa* cells took a bit longer to characterise due to its hydrophobicity. The mature pheromone consists of two 12-amino-acid peptides that differ by a single amino acid. Like *α*-factor, two genes, MFA1 and MFA2, code for a-factor. Each of these codes a single precursor that corresponds to one of the two peptides found in the mature product (Brake et al 1985). Where *α*-factor precursors are translocated to the classical secretory pathway where they are made into the mature *α*-factor (Fuller et al 1988, Kurjan 1991), a-factor secretion is very different. The a-factor precursors are very different to most precursors of secreted proteins (Kuchler et al 1989), suggesting that it is secreted by a specialised mechanism not involving the classical secretory pathway. The a-factor precursors are synthesised and processed in the cytoplasm (Schafer et al 1990, Hrycyna et al 1991). One of the proteins required for a-factor production has similar properties to proteins such as the mammalian multidrug resistance protein Mdr that spans multiple membranes (Kuchler et al 1989, McGrath & Varshavsky 1989). So it may

be that this yeast protein (Ste6) transports the a-factor across the plasma membrane.

When the opposite mating-type's pheromone is received, cells arrest at the G1 phase of the life cycle, agglutinability is induced and cells form into a pear like shape known as a shmoo (Duntze et al 1970, Bucking-Throm et al 1973, Shimoda & Yanagishima 1975, Hagiwa et al 1977, Betz et al 1978). At this point, secretion occurs through the pointed end of the shmoo in response to the pheromone (Field & Schekman 1980). This may be to localise components involved in the pheromone response and cell fusion to where the fusion will take place (Kurjan 1992). Before fusion of two cells can take place, the cell wall undergoes some changes, many of which take place at the shmoo tip (Lipke et al 1976, Lipke & Ballou 1980, de Nobel et al 1990, Osumi et al 1974). The proteins Fus1 and Fus2 help promote cell fusion (Trueheart et al 1987, McCaffrey et al 1987, Trueheart & Fink 1989) (Fig.4.).

After two cells have fused, their nuclei must then fuse into a single **a**/α nucleus. As part of the pheromone response, the spindle pole body orientates towards the shmoo tip (Byers & Goetch 1975) where the cell fusion takes place. The two spindle pole bodies are connected by microtubules that help guide the two nuclei towards each other where they will fuse. Exposure to the pheromone helps this process greatly (Rose et al 1986). The fusion of cells and nuclei results in a diploid zygote of mating type **a**/α that is capable of mitotic propagation.

Courtship in yeast was characterised by Jackson and Hartwell in 1990. They showed that cells discriminate between mating partners. Cells

producing more pheromone are more attractive and are chosen as mates more often. This suggests that there is choice for mates as seen in many higher eukaryotes. As pheromone production increases during courtship (Hagen & Sprague 1984, Hartig et al 1986, Jenness & Spatrick 1986, Strazdis & MacKay, 1983, Achestetter 1989), this choice is for an exaggerated ornament. This makes yeast an ideal system for the study of sexual selection.

1.7 Studying sexual selection in yeast

To further use *Saccharomyces cerevisiae* as a model system for studying sexual selection, the sexual display itself must first be better understood. We know how the mating pheromone is produced, secreted and subsequently processed. But why did it evolve, and why is a cell's attractiveness proportional to the amount of pheromone it produces? It is possible that the signalling display was simply a method for cells to signal their location to each other. If all cells signal equally, the strongest perceived signal will be that of the closest cell relative to the receptor. This would then allow cells closest to each other to mate and not waste time and energy in growing out unnecessarily. However such a system would only work for a limited time. Given that most mating is believed to take place within the tetrad (Tsai et al 2008), it seems odd that such a costly system of mate recognition (Smith & Greig 2010) would exist simply to make sure that matings take place between nearby cells, as most of the time a cell will be in contact with at least 2 viable potential mates.

In 1990, Jackson and Hartwell showed that strains of *S. cerevisiae* producing higher levels of mating pheromone were significantly more proficient at courtship compared to strains producing less pheromone. So should a mutation arise which increased pheromone production, then such cells would be mated with more often than expected. However, due to the cost of increased signalling, a stronger signaller could only invade a population of relatively weaker signallers if the population were under strong sexual selection pressures. Rogers & Greig showed this experimentally in 2009.

We have a theoretical start point for sexual signalling yeast, in simple mate location. And we also have a theoretical end point, with stronger signallers invading weak signallers. So we must consider how strong signallers may have evolved in the first place. This evolution depends on the cost associated with producing the pheromone. If it is costly to produce, pheromone signalling may have evolved as outlined by Fisher (1930), until the amount of pheromone produced and the level of mating success associated with it was counterbalanced with the cost of producing so much pheromone. Similarly, Pagel (1993) suggested that the yeast mating pheromone might have developed as an honest sexual signal to advertise potential mate quality as described by the handicap principle (Zahavi 1975, 1977).

While it remains unclear as to how pheromone signalling in yeast may have evolved, we do know that cells produce more pheromone than is actually needed to mate and that they increase the amount of pheromone they produce during courtship (Hagen & Sprague 1984,

Hartig et al 1986, Jenness & Spatrick 1986, Strazdis & MacKay, 1983, Achestetter 1989). This indicates that the yeast mating pheromone is indeed an exaggerated sexual trait. This makes yeast a very powerful model system for studying sexual selection. It has all the associated benefits of a microbe with respect to evolutionary research, while not only being a sexual eukaryote, but it also has mate choice for an exaggerated sexual display. Many questions need to be answered in yeast before it can be given the credibility it deserves as a model system for the study of evolution. As already mentioned, there is an unfortunate gap in our understanding of yeast ecology that hampers those who use it as a model, and it is these gaps that I try to address with this thesis. Once proper context can be applied to yeast research our level of understanding of the process of evolution will continue to increase.

1. 8 References

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1.9 Figures

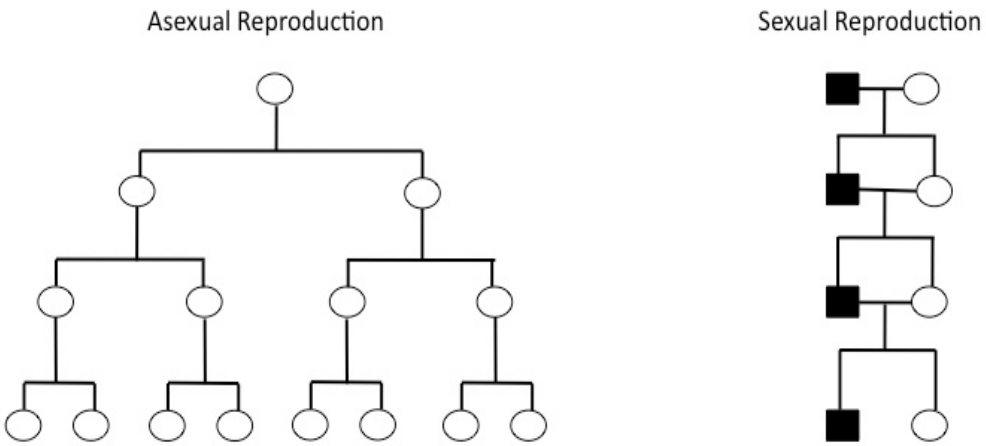

Figure 1.

The cost of males. Where each breeding couple in a sexual population can only produce an average of two offspring in their lifetime, the population will remain constant in size (right hand side). However, should a mutant parthenogenetic female appear, she will have two daughters both of which are also parthenogenetic. This new asexual line (left hand side) will quickly outcompete the sexuals (right hand side). In addition to this, asexual females will also pass on 100% of their genes, while sexual females will only pass on 50%.

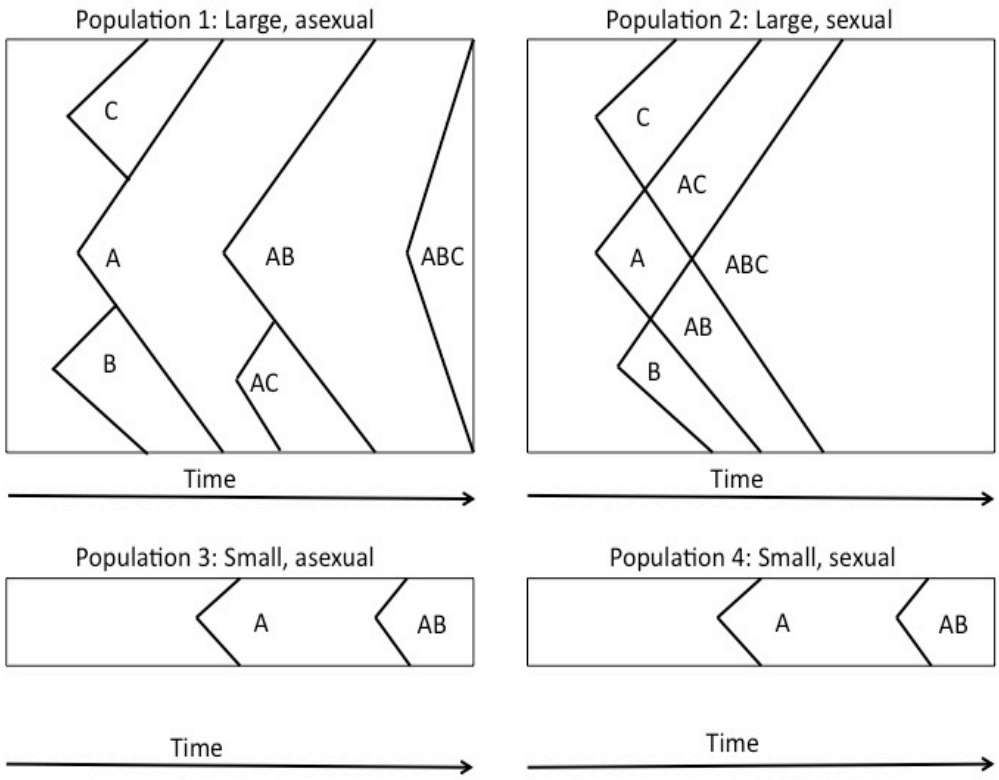

Figure 2.

Effects of recombination on rate of evolution. A, B and C represent new advantageous mutations. In the asexual populations (1 and 3), these genes can only come together until one of the mutations (e.g. B) appears in the lineage that already bears the first mutation (e.g. A). In a large sexual population (2) these mutations can be brought together much quicker through recombination. This increases the rate of adaptation. However, in small sexual populations (4), the interval between the occurence of these mutations is so long that it does not adapt any faster than an asexexual population.

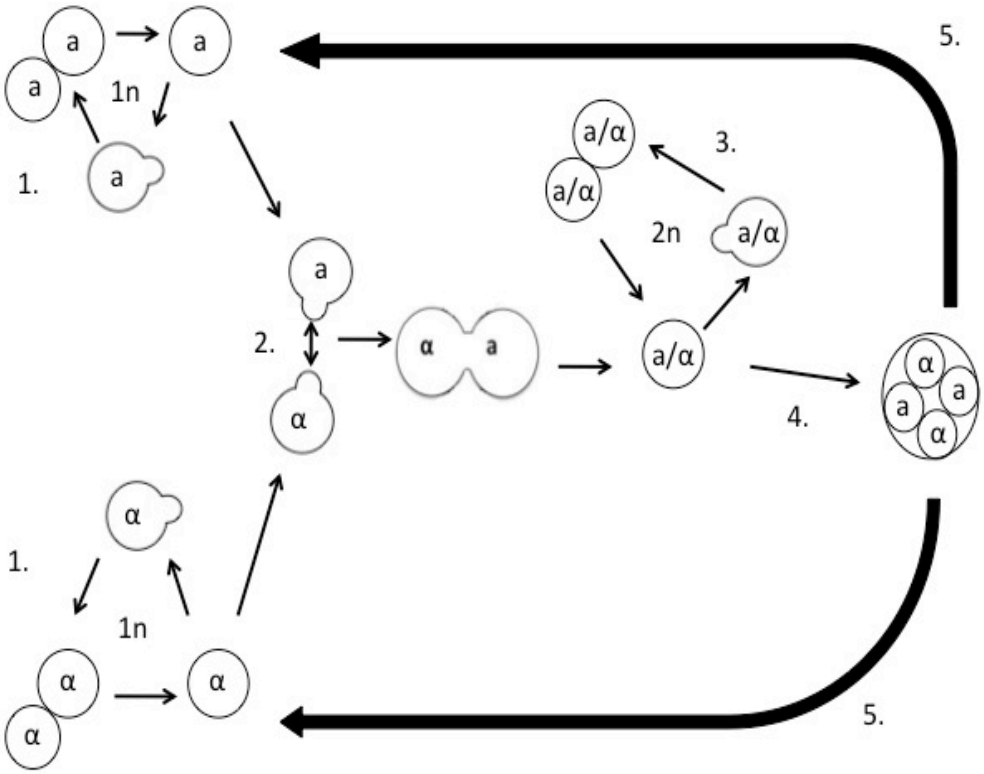

Figure 3.

The yeast life cycle. There are two haploid cell types (1n – **a** or α) and one diploid cell type (2n – **a**/α). Each of these three cell types is capable of mitotic asexual growth when there are sufficient nutrients (1 and 3). Haploid cells of opposite mating types can mate to form a dilpiod **a**/α zygote (2). When diploid cells are subject to nitrogen starvation they sporulate to form four haploid spores (two of each mating type) within an ascus (4). When these spores are introduced into an environment with sufficient nutrients, they will germinate to become vegetative haploid cells (5).

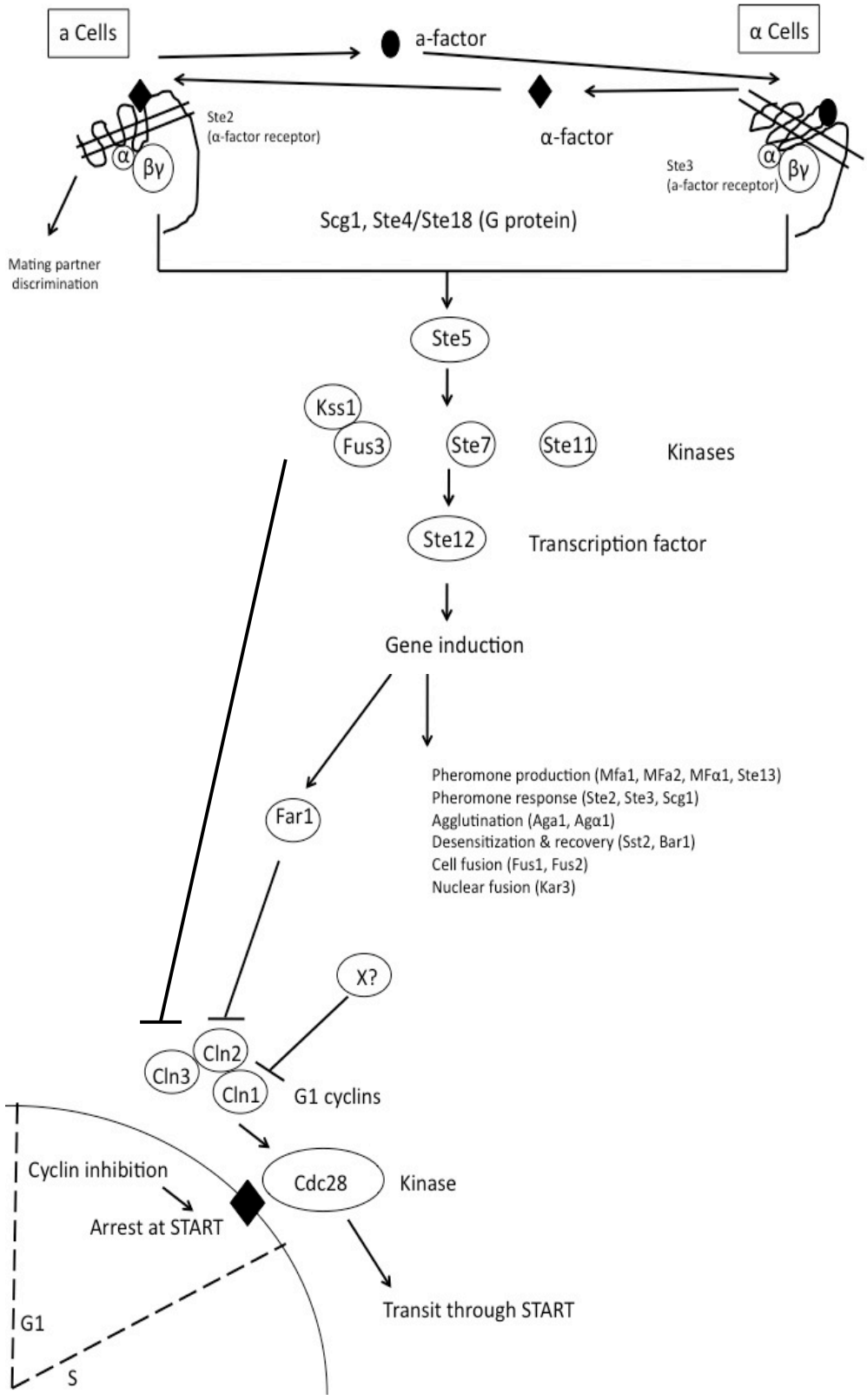
a Cells
a-factor
α Cells
Ste2
(α-factor receptor)
α-factor
Ste3
(a-factor receptor)
α
βγ
Scg1, Ste4/Ste18 (G protein)
Mating partner
discrimination
Ste5
Kss1
Fus3
Ste7
Ste11
Kinases
Ste12
Transcription factor
Gene induction
Pheromone production (Mfa1, MFa2, MFα1, Ste13)
Pheromone response (Ste2, Ste3, Scg1)
Agglutination (Aga1, Agα1)
Desensitization & recovery (Sst2, Bar1)
Cell fusion (Fus1, Fus2)
Nuclear fusion (Kar3)
Far1
X?
Cln2
Cln3
Cln1
G1 cyclins
Cyclin inhibition
Cdc28
Kinase
Arrest at START
Transit through START
G1
S

Figure 4.

Yeast pheromone response. Both cell-mating types (**a** and α) secrete mate type-specific mating pheromones (a-factor and α-factor). These pheromones bind to specific receptors (Ste3 and Ste2 respectively). A G protein composed of α (Scg1), β(Ste4) and ɣ (Ste18) subunits is thought to interact with both receptors. Downstream components necessary to activate the pathway include kinases and a transcription factor that is involved in inducing several aspects of the mating and pheromone response. Cell cycle arrest is thought to take place through inhibition of components necessary to transverse START (G1 cyclins and Cdc28 kinase). One feature of pheromone signalling between cells, is that it allows for the discrimination of potential mating partners based on their ability to produce pheromone. In **a** cells the discrimination mechanism has been shown to act through the receptor Ste2 but can occur independently of the G protein-mediated pathway.

2. General Materials and Methods

2.1 Media

Strains used throughout these experiments were maintained on media prepared to standard recipes (Burke et al 2000). Details of non-standard media are given in the relevant chapters. Solid and liquid media are identical except for the absence of agar in the liquid versions. Constituents were obtained from the following sources: Yeast extract (Merck), peptone and glucose (Fisher scientific), glycerol and potassium acetate (VWR), agar and yeast nitrogen base without amino acids (Difco) and individual amino acids (Sigma). G418 media was made by adding 200-400mg/l of G418 (Fisher) to the standard YEPD media after autoclaving.

2.2. Enzymes

Taq DNA polymerase (Eppendorf), Proteinase K (Fisher), RNase A (Sigma) and Zymolyase 20T (USBiological) were all used as instructed by the manufacturer.

2.3 Strains

2.3.1 Saccharomyces cerevisiae

Derivatives of the strain Y55 are used most commonly in this work. Y55 was most likely originally isolated from a French grape by Ojvind Winge between 1930 and 1960 (Greig 1999). The auxotrophic markers used were made at the Haber laboratory by mutagenesis, or in the Yeast Genetics Laboratory (Oxford) through 2-step gene replacement or transplacement (Greig 1999).

S288c is also used in places in this work. The genetic background of this strain is unknown but it has been shown, by developing a pedigree based on

early studies, that 88% of the gene pool can be traced to a wild isolate obtained from a rotting fig in 1938 in California (Mortimer & Johnston 1985).

2.3.1 Saccharomyces paradoxus

All *S. paradoxus* strains used in this work are derived from the strain N-17. It was first isolated from a Russian oak tree by Naumov et al (1988) and was later made heterothallic by Chambers et al (1996). Many auxotrophic markers were produced by Hunter et al (1996) using UV mutagenesis. Duncan Greig (1999) also isolated *lys2*, *lys5*, and *ura3* mutants from the original isolate.

2.4 Genomic DNA preparation

The glass bead extraction method described by Burke et al (2000) was used for extracting DNA, over longer protocols as it produced DNA of sufficient quality for the few PCR reactions required. Cells were suspended in 200µl of glass bead solution (1% SDS, 1mM NaCl, 10mM Tris-Cl (pH8), 1mM Na^2 EDTA). 200µl of phenol:chloroform:isoamyl alcohol (25:24:1) along with 0.3g of acid washed 0.5mm glass beads was then added. This mixture was then vortexed for 2 minutes to break up the cells. This was centrifuged for 5 minutes, after which the top layer of the mixture was removed and placed into a clean eppendorf tube. 1ml of ethanol was added and mixed in by inverting the eppendorf. The new mixture was then centrifuged for 10 minutes to pellet the DNA. The ethanol was removed and the pellet was air drief before being suspended in 70µl of TBE (Tris/Borate/EDTA buffer) (pH8). The DNA was then stored at -20℃.

2.5 PCR

PCR primers were designed using the *Saccharomyces* genome database (http://www.yeastgenome.org/).

20µl PCR reactions were used (2µl 10X Tap buffer advanced (eppendorf), 0.5µl 10mM dNTP mix (Q-Biogene), 0.06µl (0.3 units) Tap DNA polymerase (eppendorf), 15.5µl purified H_2O (Sigma), 1µl of 10µM F+R primer mix and 1µl of 1/100 diluted genomic DNA prep). The reaction cycle was done using a MJ research PTC-200 thermal cycler set to the following program:

1. 96°C for 1 minute
2. 96°C for 30 seconds
3. 48°C for 30 seconds
4. 72°C for 30 seconds
5. cycle from step 2 – x 34
6. 72°C for 5 minutes

Products were visualised by gel electrophoresis on a 1.5% agarose gel (1 x TBE) containing ethidium bromide at 70V using a Scie-Plas HU20 gel tank and Consort E132 power supply. A UV transilluminator (UVP) and a DigiDoc-It Darkroom and digital camera (UVP) were used to record gels.

2.6 Transformation

Transformations were carried out as described by Gietz & Woods (2002). Cells to be transformed were inoculated into 5ml liquid YEPD and incubated over night. 0.4ml of this culture was then transferred into a fresh tube of 5ml

YEPD and incubated for a further 4 hours. This is to ensure the cells are optimised for efficient transformation. Following the 4 hour incubation, the culture was split evenly into 4 eppendorf tubes. These were then centrifuged and the supernatant removed. 1ml of sterile water was added to two of the eppendorfs, the cells were resuspended and then transferred to the two eppendorfs with pelleted cells, such that the original culture was now split. Both eppendorfs were vortexed to make sure all cells were in suspension. Both suspensions of cells underwent the transformation process, but one acted as a control and is not mixed with the sequence to be added. Both tubes were centrifuged to pellet the cells. The supernatant was removed and the transformation mixture was added in the following order: 240µl polyethylene glycol (MW 3350) 50% w/v, 36µl 1M lithium acetate, 50µl boiled single stranded carrier DNA and 34µl of the transformind DNA solution (36µl of sterile water was used here for the control cells). Both tubes were then vortexed and heat shocked for 20 minutes. The eppendorfs were then centrifuged at low speed for 30 seconds. The supernatant (transformation mixture) was removed and 1ml of sterile water was added to the pellet of cells. This was then mixed by vortex to wash the cells. The water was removed from the cells (by centrifuge and pipette) before being resuspended in 100µl of sterile water. The cells were then spread onto the relevant selective media plate and incubated for 4 days at 30℃ after which successful transformants were selected.

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3. The Cost of Sexual Signalling in Yeast

3.1 Abstract

The handicap principle holds that a sexual signal can act as a reliable indicator of mate quality if it is costly to produce. The cost assures that only individuals of high quality can afford to produce a strong signal - the cost of signaling is relatively lower for high quality signalers than for low quality signalers. This critical property is difficult to test experimentally because the benefit of signaling on mating success, and cost of signaling on other components of fitness, cannot easily be separated in obligate sexual organisms. We therefore studied the facultatively sexual yeast *Saccharomyces cerevisiae*, which produces pheromones to attract potential mates. To precisely measure the cost of signaling, the signal was reduced or removed by deleting one or both copies of the pheromone-encoding genes and measuring asexual growth rate in competition with a wild-type signaler. We manipulated signaler quality either by changing the quality of the assay environment or by changing the number of deleterious mutations carried. For both types of treatment, we found that the cost of signaling decreased as the quality of the signaler increased, demonstrating that the yeast pheromone signal has the key property required for selection under the handicap principle. Finally, we verified that cells of high genetic quality do indeed produce stronger signals than low quality cells, showing that the signal acts as an honest signal of quality.

3.2 Introduction

The handicap hypothesis of sexual selection (Zahavi 1975; Anderson 1994; Maynard Smith & Harper 2003) proposes that exaggerated sexual displays evolve because they accurately convey the quality of potential mates. Strong signalers are preferred as mates because they provide more benefits to the receiver than weak signalers. These benefits can be direct, in the form of investment in the production of offspring, or indirect, in the form of good genes that are transmitted to the offspring. Thus the strength of the sexual signal must be dependent on the phenotypic condition or the genetic quality of the signaler. In many species, there is good evidence that the size of sexual displays is positive correlated with phenotypic quality, but there are few studies in which quality is manipulated experimentally to determine its effect on signaling (Cotton et al. 2004).

Under the handicap principle, a sexual display acts as an honest signal – a reliable indicator of its bearer's quality – because it is costly to make or maintain. This cost, which actually reduces the fitness of the signaler, prevents low quality individuals from falsely advertising high quality because they cannot afford to. On the other hand, high quality individuals can better afford to produce larger sexual signals and so are identified and preferred as mates because of the direct or indirect benefits they convey. The cost of signaling is therefore critical to the handicap hypothesis, but it is difficult to measure in most study organisms. Darwinian fitness can potentially be determined from the

number of offspring left in future generations, but an honest signal that reduces fitness because of the cost of signaling should simultaneously increase fitness by ensuring that the carrier has greater mating success. Thus isolating the cost of signaling from its reproductive benefit requires the measurement of viability, "all components of fitness other than mating success" (Maynard Smith 1987). Although the effects of sexual displays on individual fitness components such as energy expenditure can be determined (Nicoletto 1991; Saino et al. 1997; Basolo & Alcaraz 2003), this is not sufficient to show they affect viability – costs in one trait may be outweighed by benefits in another, unmeasured, trait (Kirkpatrick 1987). Furthermore, the sexual signal must not only be costly, but the cost must be relatively lower for higher quality signalers and higher for lower quality signalers (Maynard Smith & Harper 2003; Kotiaho 2000). In most organisms studied for sexual selection, testing this critical property experimentally is very difficult, and, to date, there are only two examples. In the barn swallow, males manipulated to display larger signals (by artificially extending their tails) survived less well than males with naturally long tails (Møller & de Lope 1994). In the wolf spider, males in poor condition that were provoked to signal more than usual did not live as long as males in good condition that were subjected to a similar treatment (Kotiaho 2000). Here we take advantage of the fact that yeast can grow both sexually and asexually to measure the cost of sexual signaling by the reduction in asexual fitness that signaling causes. Asexual fitness is a good measure of viability, as it excludes fitness components related to mating, but includes the other fitness components of the life

cycle. We measure relative asexual fitness directly by competition assays between pairs of strains (Lenski et al. 1991).

The life cycle of yeast is well defined under laboratory conditions, but it is poorly understood in the wild. Nevertheless, it seems likely that yeast usually reproduces by mitosis as an asexual diploid which can occasionally enter meiosis to produce haploid cells (Leu & Greig 2009). Two mating-types of haploid cell are produced, defined by the allele, *MATa* or *MATα*, carried at the mating-type locus. This allele determines gene expression differences between the two types of haploids. *MATa* cells secrete a-pheromone and produce cell surface receptors specific to α-pheromone. *MATα* cells secrete α-pheromone and produce cell surface receptors specific to a-pheromone. Like diploids, haploids can divide by mitosis indefinitely, which enables asexual fitness assays to be carried out between competing haploid strains of the same mating type. But if haploids detect the pheromone of the other mating type, they stop dividing and send out a cellular projection towards the source of the pheromone. In this way, cells of the opposite mating-type touch each other and fuse together, producing new diploids. Diploids are therefore always heterozygous at the mating-type locus, and do not produce or detect either type of sex pheromone.

Sexual signaling and mating in yeast has been well studied. Haploid cells use a sensitive and precise signal transduction system to detect and respond to the pheromone of the other mating type (Yu et al. 2008). The pheromone signal strength can be manipulated genetically

because the two pheromone types are each encoded by two semi-redundant genes: *MFα1* and *MFα2* produce α-pheromone and are only expressed in *MATα* cells, and *MFa1* and *MFa2* produce a-pheromone and are only expressed in *MATa* cells. Cells that lack one of the pheromone-encoding genes produce less pheromone but can nevertheless mate normally (Jackson & Hartwell 1990, Rogers & Greig 2009) but loss of both copies prevents signaling and mating altogether, causing sterility (Sprague & Thorner 1992). The genetic tractability of this mating system has previously been exploited to demonstrate the process of sexual selection in a laboratory (Rogers & Greig 2009).

The production of yeast mating pheromones, and the responses to them, may have originated as a system to improve mating efficiency. If all cells signal at the same level, then cells preferring the strongest source would benefit by selecting the nearest partner, a system known as passive attraction. But such a system should be evolutionarily unstable (Pagel 1993) because, given the choice between two equidistant signalers, cells actively choose the one producing more pheromone (Jackson & Hartwell 1990). So mutations that increase pheromone production would be expected to spread and the mating efficiency benefits of preferring the strongest pheromone source would be lost because the strongest source would not necessarily be the closest mate but rather the strongest signaler. But Pagel (1993) showed that if the pheromone signal was costly to produce, it could evolve under the handicap principle to signal mate quality. The preference for stronger signalers would remain beneficial,

because stronger signalers would be better quality mates. The fact that cells produce pheromone at a higher level than required for mating (Sprague & Thorner 1992), and that they increase their pheromone signal further when they detect the pheromone of the other mating type, suggests that the pheromone signaling system has not evolved just to find the nearest mate but also to find the best mate (Pagel 1993).

3.3 Materials and Methods

3.3.1 Asexual fitness assays

Relative viability (asexual fitness) was measured by direct competitions between pairs of strains that could be distinguished using a genetic marker. Competing strains were first streaked on YEPD-agar (1% yeast extract, 2% peptone, 2% dextrose, 2.5% agar) and a single colony from each was used to inoculate separate tubes containing 5ml of liquid assay medium. The two tubes were incubated for 24 hours with shaking in the assay conditions and then equal volumes were mixed together and 50μl of this mixture was used to inoculate a fresh tube of liquid assay medium. A sample was serially diluted, plated onto YEPD-agar to yield single colonies, and these colonies were replica-plated to the appropriate selective medium to distinguish between the two strains, to determine the initial numbers of each strain in the mixed population. The tube containing the mixed population was incubated for 24 hours and then a second sample was taken and plated as before to determine the final numbers of each strain after asexual growth in the assay environment.

The fitness of each strain was calculated relative to the other by the ratio of their Malthusian parameters (Lenski et al. 1991).

3.3.2 Effect of pheromone gene deletion on viability

The effect of signaling on asexual fitness was determined by performing asexual fitness assays between a wild-type signaler and a strain with a reduced or removed signal. Five assays were performed each between strains YDG708 (*MATα his3 leu2 ura3 lys2 met15*) and YCS66 (*MATα mfα1::KanMX4 his3 leu2 ura3 lys2 met15*), between YDG710 (*MATa his3 leu2 ura3 lys2 met15*) and YCS68 (*MATa mfa1::KanMX4 his3 leu2 ura3 lys2 met15*), between YDG708 (*MATα his3 leu2 ura3 lys2 met15*) and YDG707 (*MATα mfα1::KanMX4 mfα2::KanMX4 his3 leu2 ura3 lys2 met15*), and between YDG710 (*MATa his3 leu2 ura3 lys2 met15*) and YDG709 (*MATa mfa1::KanMX4 mfa2::KanMX4 his3 leu2 ura3 lys2 met15*). These strains are isogenic with the laboratory strain S288c (Mortimer & Johnstone 1986). The assay medium was liquid YEP-Gal (1% yeast extract, 2% peptone, 2% galactose) supplemented with 0.5M salt and incubated at 35°C with shaking, and the selective medium used to distinguish between the two strains in each competition was G418-agar (as YEPD-agar but supplemented with 0.04% G418 antibiotic), on which only strains carrying the *KanMX4* marker can grow. The effect of deleting pheromone genes on signal strength was visualized by making patches of the strains on YEPD-agar, incubating at 30°C for one day, spraying with a

tester strain hypersensitive to either α-pheromone or a-pheromone, and incubating again for another day. The tester strains were YDG1065 (*MATα ade2 leu2 met15 ura3 cyh2r his3 sst2::KMX*) and YDG1121 (*MATa leu2 met15 ura3 his3 sst2::KMX bar1::URA3*), both isogenic with S288c.

3.3.3 Effect of phenotypic quality on cost of signalling

The effect of different environmental conditions on phenotypic quality of wild-type signalers YDG708 (*MATα*) and YDG710 (*MATa*) was measured using a Biowave CO8000 cell density meter (Biochrom, Cambridge, UK). These two strains were streaked onto YEPD-agar and a single colony was used to initiate cultures in eight different environmental conditions: YEPD at 30°C, YEPD at 35°C, YEPD supplemented with 0.5M salt at 30°C, YEPD supplemented with 0.5M salt at 35°C, YEP-Gal at 30°C, YEP-Gal at 35°C, YEP-Gal supplemented with 0.5M salt at 30°C, and YEP-Gal supplemented with 0.5M salt at 35°C. Tubes were incubated for 24 hours with shaking and samples of each culture were transferred into fresh medium in the same conditions for a further 4 hours after which hourly OD600 measurements were taken for eight hours. The measurements were plotted against time and the log-transformed slope was used as a measure of phenotypic quality. The effect of phenotypic quality on the viability cost of signaling was determined by performing asexual fitness assays between wild type *MATα* signaler YDG708 and *MATα* non-signaler YDG707, and between

wild type *MATa* signaler YDG710 and *MATa* non-signaler YDG709 under each of the eight environmental conditions. The marker *KanMX4*, carried only by the non-signalers, was used to distinguish between the two strains as before.

To test the generality of this result, a smaller experiment was also performed using a different genetic background, Y55 (McCusker & Haber, 1988). Wild type signaler YCS50 (*MATα ura3*) was competed against weak signaler YCS54 (*MATα mfα1::KanMX4 ura3*) in five replicate fitness assays each in two environments, high quality (YEPD at 30°C) and low quality (YEP-Gal supplemented with 0.5M salt at 35°C). The quality of the environments was quantified as before but using YCS50.

3.3.4 Effect of genetic quality on cost of signalling

The effect of different numbers of mutations (actually auxotrophic markers) on genetic quality was determined with competitive asexual fitness assays. Five asexual fitness assays each were done between YCS50 (*MATα ura3*) and YCS73 (*MATα ade2 ura3*), between YCS50 and YCS45 (*MATα lys2 his4 leu2 thr4 met13 can1r ade2 ura3*), between YCS50 and YCS176 (*MATα ura3 lys2 ade1::KanMX4*) and between YCS50 and YCS174 (*lys2 his4 leu2 thr4 met13 can1r ura3 ade1::KanMX4*). The assay medium was YEPD at 30°C, and the selective medium, used to distinguish between the competing strains, was adenine-dropout-agar (2% glucose, 0.54%

yeast nitrogen base, 2% agar, 0.087% amino acid mix containing all required amino acids except adenine).

Having measured the genetic quality of strains carrying five different combinations of deleterious mutations, we next measured the cost of signaling in these five different genetic backgrounds. We did five asexual fitness assays each between YCS50 (*MATα ura3*) and YCS53 (*MATα ura3 mfα1::URA3*), between YCS73 (*MATα ade2 ura3*) and YCS75 (*MATα ade2 ura3 mfα1::URA3*), between YCS45 (*MATα lys2 his4 leu2 thr4 met13 can1r ade2 ura3*) and YCS49 (*MATα lys2 his4 leu2 thr4 met13 can1r ade2 ura3 mfα1::URA3*), between YCS176 (*MATα ura3 lys2 ade1::KanMX4*) and YCS177 (*MATα ura3 lys2 ade1::KanMX4 mfα1::URA3*), and between YCS174 (*lys2 his4 leu2 thr4 met13 can1r ura3 ade1::KanMX4*) and YCS175 (*lys2 his4 leu2 thr4 met13 can1r ura3 ade1::KanMX4 mfα1::URA3*). As a control to test the neutrality of the *URA3* marker used to delete *MFα1*, we also performed five fitness assays each between YCS50 (*MATα ura3*) and YCS64 (*MATα*), between YCS73 (*MATα ade2 ura3*) and YCS74 (*MATα ade2*), between YCS45 (*MATα lys2 his4 leu2 thr4 met13 can1r ade2 ura3*) and YCS65 (*MATα lys2 his4 leu2 thr4 met13 can1r ade2*), between YCS176 (*MATα ura3 lys2 ade1::KanMX4*) and YCS179 (*MATα lys2 ade1::KanMX4*), and between YCS174 (*lys2 his4 leu2 thr4 met13 can1r ura3 ade1::KanMX4*) and YCS178 (*lys2 his4 leu2 thr4 met13 can1r ade1::KanMX4*). The assay medium was YEPD at 30°C, and the selective medium, used to distinguish between the competing strains, was uracil-dropout-agar (2% glucose, 0.54% yeast nitrogen base, 2% agar, 0.087%

amino acid mix containing all required amino acids but not uracil). These strains are all isogenic with the laboratory strain Y55 (McCusker & Haber, 1988).

3.3.5 Effect of genetic quality on signalling strength

The *MAT*α strains YCS50 and YCS174 (described above), as well as isogenic strains in the other mating type YCS1 (*MAT*a *ura3*) and YCS188(*MAT*a *lys2 his4 leu2 thr4 met13 can1r ura3 ade1::KanMX4*), were grown in 5ml of liquid YEPD with shaking for 24 hours. A sample of each was serially diluted and plated onto YEPD-agar to yield single colonies. Plates for fit strains YCS1 and YCS50 were incubated for 24 hours; plates for unfit strains YCS188 and YCS174 were incubated for 38 hours. This was to produce colonies containing similar numbers of cells across all 4 strains, despite the differences in fitness. Colonies were then punched out from the agar plates using the wide end of a sterile 200μl pipette tip. The resulting agar plates (with signaller colonies removed), were sprayed with a suspension of tester strains sensitive to a-pheromone or α-pheromone (YDG1065 and YDG1121, described above). The punched out signaller colonies in the meantime were suspended in 1ml of water and then serially diluted and plated onto fresh YEPD-agar to measure the number of cells per colony.

The sprayed plates formed lawns of tiny colonies from the sprayed tester strains, which covered the surface of the agar except where pheromone inhibited their growth, forming “haloes” (like those

shown in Fig. 1.). The plates were photographed digitally, and open source software ImageJ was used to measure the area of each "halo", giving a measure of strength of pheromone signal strength of each of the previously punched out colonies.

3.4 Results

3.4.1 The cost of signalling

Deleting one or both pheromone-encoding genes reduces or removes the pheromone signal (Fig. 1.). We measured the viability cost of producing α- and **a**-pheromone signals by determining the asexual fitness advantage of having a reduced or a removed signal in competition with wild-type strains that had both pheromone encoding genes intact (Fig. 2. and Table S1 Supporting Information). A two factor ANOVA (Table S2 Supporting Information) showed that both the number of pheromone genes deleted ($F_{1,16}$=14.08, p=0.0017) and the pheromone type ($F_{1,16}$=17.38, p=0.0007) had significant effects on viability. There was no significant interaction between these factors ($F_{1,16}$=0.92, p=0.3509), thus the cost of increasing investment in the signal is independent of which type of pheromone is produced. Signaling is costly, and increasing investment in the signal increases the cost.

3.4.1The effect of signaler quality on the cost of signalling

The key property of a sexual signal evolving under the handicap principle is that the relative cost of signaling becomes lower as the quality of the signaler improves. To test this we again measured the viability of non-signalers (with both genes encoding a pheromone deleted) relative to signalers (with intact pheromone-encoding genes), but we also manipulated phenotypic condition by carrying out the fitness assays under different quality environments. The effect of different environments on quality was measured by the log phase asexual reproduction rate of the signaler in the absence of competition (see Materials and Methods), and the cost of signaling on viability was measured by competition between signalers and non-signalers in the different environmental conditions. Fig. 3. shows how the asexual fitness of non-signalers relative to wild-type signalers (the cost of signaling) is reduced as phenotypic quality increases. ANCOVA of the data (Supporting Information Tables S3 and S4) showed that both signaler quality ($F_{1,64}$=286.95, p<0.0001) and pheromone type ($F_{1,64}$=36.05, p<0.0001) had significant effects on the viability cost of signaling. The cost of producing a pheromone decreases significantly as quality increases (slope= -0.68, t= -10.30, p<0.0001); for α-pheromone this decrease is significantly steeper (slope= -1.27, t=-5.49, p<0.0001). The generality of this result was confirmed by a smaller experiment with a different genetic background (Supporting Information Table S5). The cost of signaling was again found to be higher in a low quality environment than in a high quality environment (t=4.06, df=6, p=0.003). Thus individuals in good condition pay a lower cost of signaling than low quality individuals.

Next, we tested whether the relative cost of signaling depended also on genetic quality, as well as phenotypic quality (Fig. 4). We measured the asexual fitness in a strain with a reduced signal relative to a normal signaler, in five different genetic backgrounds. The different genetic backgrounds contained different numbers of loss-of-function mutations which reduced fitness to 0.89 (n=5, SD=0.0057), 0.76 (n=5, SD=0.0049), 0.68 (n=5, SD=0.005) or 0.57 (n=5, SD=0.0033) relative to the fittest background (nominal fitness of 1.0). The cost of signaling was measured by competing strains that had the same genotypes, apart from at the locus controlling signaling. Genetic quality had a significant effect on the viability cost of signaling (Supporting Information Table S6, r^2= 0.8353, DF=24, p<0.0001). As a control, we also measured the fitness effect of the marker *URA3*, but found this was not affected significantly by the five genetic backgrounds (Supporting Information Table S6, r^2= 0.0297, DF=24, p=0.4100).

3.4.3 The effect of genetic quality on signalling strength

Finally, we confirmed that better quality cells do in fact signal more strongly than poor quality cells. We used the fittest and least fit *MAT*α signaller strains from the previous experiment (see above, and Fig. 4.), as well as *MAT*a signaller strains carrying the same combinations of deleterious mutations. These mutations reduced competitive asexual fitness of the low quality signaller to 0.57 (n=5, SD=0.0033) relative to the high quality signaller. The signal strength of these strains is shown in Fig. 5. (data in Supporting Information Tables S7 and S8). ANCOVA of the data

(Supporting Information Table S9) shows that the signal strength of *MAT*α colonies depends on the asexual fitness of the signaller cells ($F_{1,24}$=180.42, p<0.0001), when the number cells per colony is accounted for ($F_{1,24}$=157.23, p<0.0001). When the interaction between fitness and cell number was included, it was not significant ($F_{1,24}$=0.18, p=0.6758), so it was removed from the model. Likewise (Supporting Information Table S10), *MAT*a asexual fitness also determines signal strength ($F_{1,16}$=66.28, p<0.0001) when cell number ($F_{1,16}$=61.24, p<0.0001) is accounted for. There was no significant interaction between fitness and cell number ($F_{1,16}$=0.10, p=0.7564), so the interaction term was removed from the model. These data confirm that high quality strains signal more strongly than low quality strains – the pheromone is an honest indicator of genetic quality.

3.5 Discussion

We have shown that the yeast sex pheromone is costly to produce, capable of reducing viability by over 30% (Fig. 2.). But this cost depends on both the phenotypic and genotypic quality of the signaler (Fig. 3. and Fig. 4.), with high quality signalers paying a much smaller cost than low quality signalers. As expected, low quality signalers produce weaker signals than high quality signalers under the same experimental conditions (Fig. 5.). Even if the signaling system originated for passive attraction (i.e. to locate the nearest mate), these qualities mean it is expected to evolve as an honest signal, in accordance with the handicap principle (Pagel 1993).

It is remarkable that deleting just one or two genes improves asexual fitness so dramatically, but this high cost of signaling is exactly what is expected to evolve under the handicap principle. It is consistent with the longstanding article of yeast laboratory folklore that haploids propagated asexually lose the ability to mate because mutations eliminating the signaling pathways are rapidly selected. This phenomenon was investigated experimentally by Zeyl et al. (2005), who identified an evolutionary trade off between mating ability and asexual viability, perhaps caused by the high cost of signaling. It is not clear what the physiological basis is for the high signaling cost, because production of pheromone is a complex process. The precursor of α-pheromone, which contains tandem repeats of the mature peptide, is imported into the endoplasmic reticulum where asparagine-linked oligosaccharides are attached (Sprague & Thorner 1992). These oligosaccharides are extended as the precursor passes through the Golgi apparatus before it is cleaved to release the pheromone peptides that are further processed before secretion. Production of the a-pheromone is quite different, as the precursor is not processed in the Golgi apparatus, but is instead modified extensively in the cytosol and on the inner face of the cytoplasmic membrane before the mature peptide is secreted by a novel mechanism (Sprague & Thorner 1992). In our study, α-pheromone was more costly to produce than a-pheromone (Fig. 2. and Fig. 3.), but this may well be a consequence of the fitness assay conditions, which probably do not represent the conditions or stresses that yeast encounter in nature.

Although yeast is the best understood eukaryote at the cellular and molecular level, we know very little about its natural life (Replansky et al. 2008, Leu & Greig 2008). Recent population genetics (Tsai et al. 2008) and phylogenetic (Ruderfer et al. 2006) studies of yeast outside the laboratory have made some surprising findings, including the fact that most mating occurs between haploids formed from the same meiosis, resulting in automixis. This is further evidence against the passive attraction hypothesis – why would haploids need a diffusible pheromone system to find mates, when suitable mates from their own meiosis are right next to them? If instead the pheromone signals haploid quality, then it might help clear recessive deleterious mutations, accumulated during the previous diploid phase but exposed to selection in the haploid phase, by allowing the two fitter (least mutated) haploids from a meiosis to mate and found the next diploid phase. Studying pheromone signaling in yeast under natural conditions is difficult, but the unprecedented ability to genetically manipulate signal strength, combined with the precise measurement of viability by competitive growth assays, makes yeast a promising model organism for studying sexual selection theory. Sexual selection is not just about beautiful traits in animals, birds, and plants, but it plays an important part in the evolution of tiny microbes too.

3.6 References

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3.7 Figures

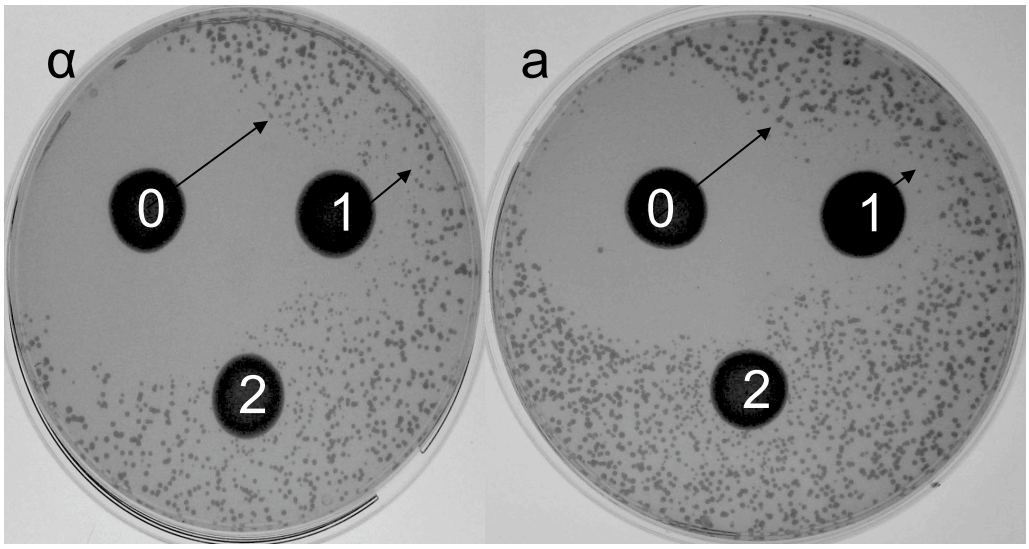


Figure 1.

Effect of pheromone gene deletion on signal strength.

Visualization of signal strength for α-pheromone (left) and a-pheromone (right). Circular patches of yeast cells labeled 0, 1, or 2 contain strains with 0, 1, or 2 pheromone-encoding genes deleted. After the patches were grown up, the plates are sprayed with a light lawn of tester strain hypersensitive to the appropriate pheromone (see Materials and Methods), which forms small colonies in the background unless inhibited by production of the pheromone from the patches. The zone of inhibition or "halo" is indicated by the radial arrows from the centre of the patches. On the left hand plate, the patch labelled 0 is *MATα* wild type signaler YDG708, the patch labelled 1 is *MATα* weak signaler YCS66, and the patch

labelled 2 is *MATα* non-signaler YDG707. On the right hand plate, the patch labelled 0 is *MATa* wild type signaler YDG710, the patch labelled 1 is *MATa* weak signaler YCS68, and the patch labelled 2 is *MATa* non-signaler YDG709.

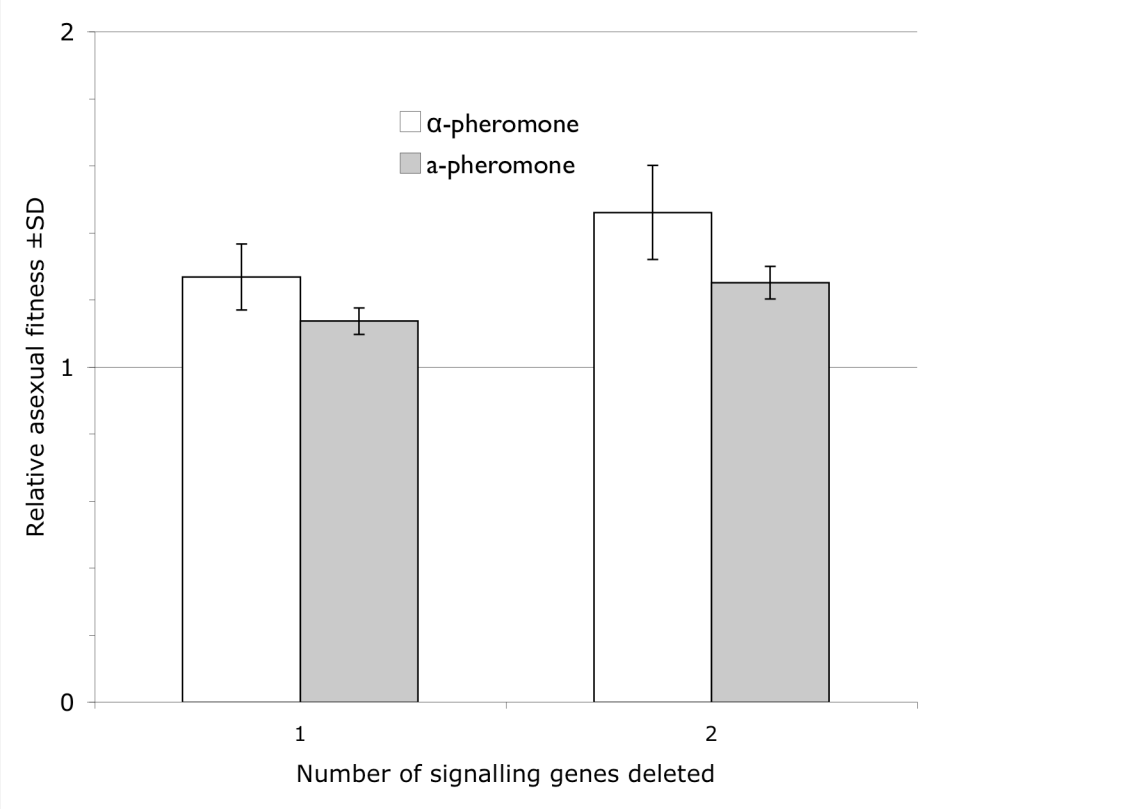


Figure 2.

Effect of pheromone gene deletion on viability. Asexual competitive fitness of strains with reduced (1 pheromone gene deleted: YCS66 *MATα,* YCS68 *MATa*) or removed (2 pheromone genes deleted: YDG707 *MATα,* YDG709 *MATa*) signals, relative to isogenic wild-type signallers (YDG708 *MATα*, YDG710 *MATa***)**. Reducing pheromone production increases viability, and removing it increases viability further. The cost of signalling depends on the strength of the signal.

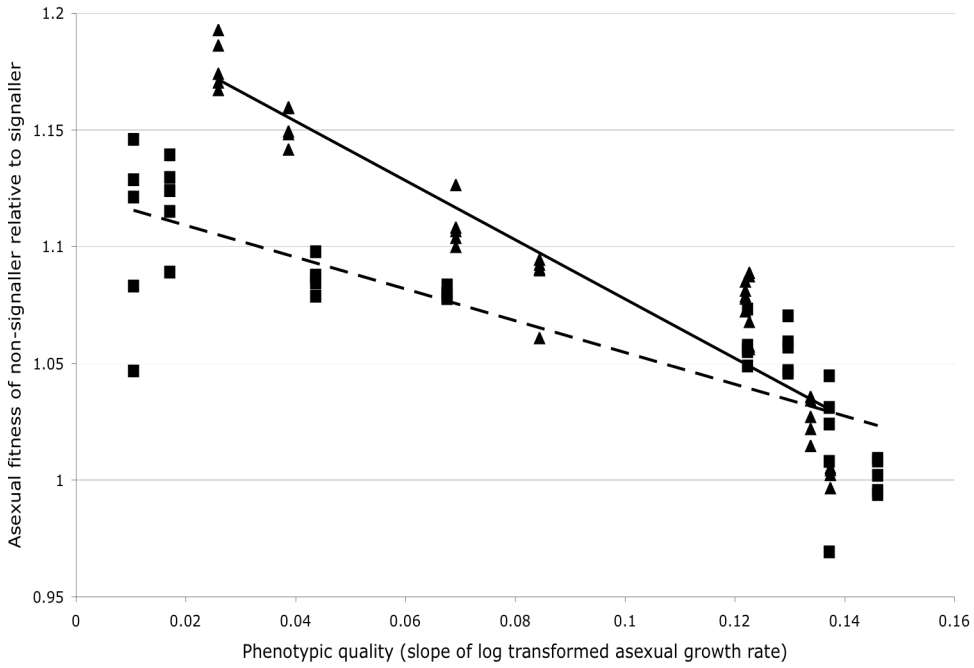


Figure 3.

The cost of signalling depends on the phenotypic quality of the signaler. The effect of phenotypic quality on the cost of signaling is shown by the relative fitness advantage of non-signalers (2 pheromone genes deleted: YDG707 *MATα,* YDG709 *MATa*) relative to isogenic wild type signalers (YDG708 *MATα*, YDG710 *MATa***)** under different environmental conditions. The quality of the environments was measured by the log of the exponential growth rate of the signalers. Triangles and solid line indicate data for α-pheromone, squares and dashed line for a-pheromone.

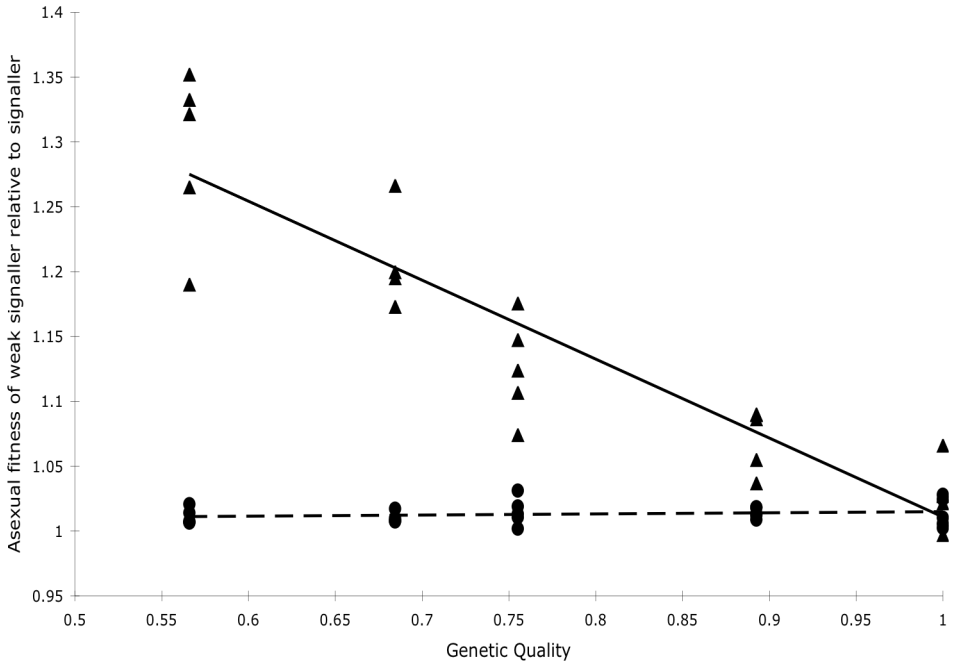
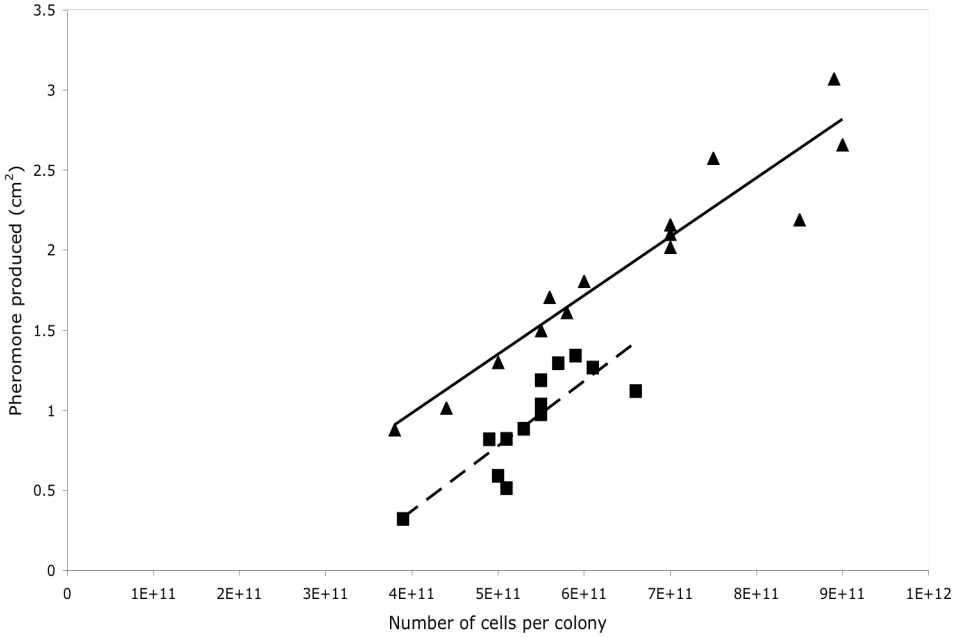


Figure 4.

The cost of signaling depends on the genetic quality of the signaler. The effect of deleterious mutations on the cost of signaling is shown by the relative fitness advantage of *MATα* weak-signalers relative to isogenic wild type *MATα* signalers in five genetic backgrounds differing in the composition of deleterious mutations carried. The quality of the different genetic backgrounds was determined by asexual competition of a wild type signaler from each relative to the wild type signaler from the least-mutated background (fitness of 1). The triangles and solid line shows how the viability cost of signaling becomes less as the signaler quality improves. As a control, we also measured the fitness effect of the marker *URA3* in wild-type signalers in the different genetic backgrounds (circles, dashed line).

a)



b)

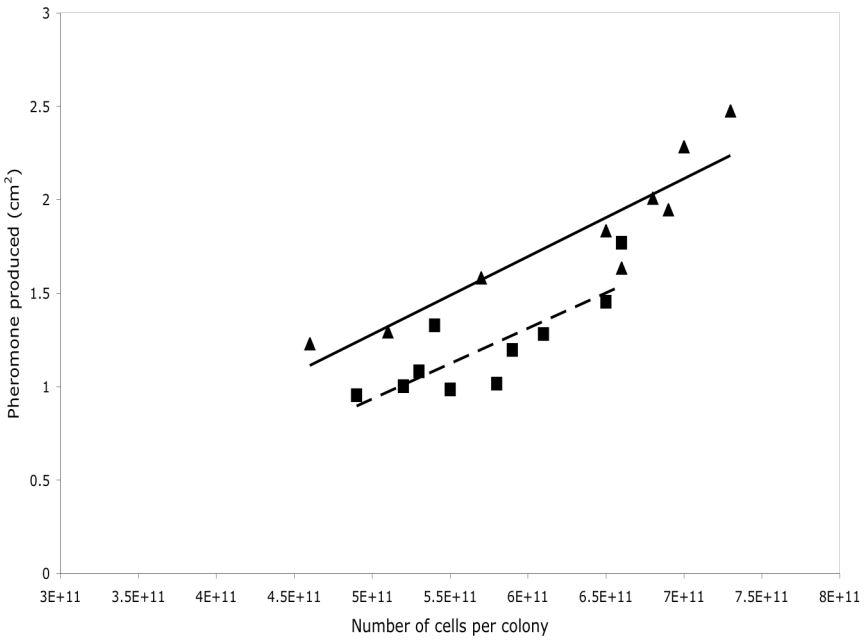


Figure 5.

Effect of genetic quality on signal strength. 5a shows the effect of signaller quality on a-pheromone signal strength, 5b shows this for α-pheromone. Good quality cells (triangles, solid line), carrying few deleterious mutations and with high asexual fitness, produce stronger

signals than poor quality cells (squares, dashed line) which carry more deleterious mutations and have lower asexual fitness. Signal strength was quantified by measuring the areas (cm^2) of the "haloes" of inhibition (similar to those shown in Figure 1) produced by colonies of known numbers of signalling cells. The assay is described in Materials and Methods.

3.8 Supporting Information

Strain	Mating type	Genes deleted	Fitness	Fitness - 1
YDG708 vs YCS66	α	1	1.163	0.163
YDG708 vs YCS66	α	1	1.170	0.170
YDG708 vs YCS66	α	1	1.386	0.386
YDG708 vs YCS66	α	1	1.330	0.330
YDG708 vs YCS66	α	1	1.290	0.290
YDG708 vs YDG707	α	2	1.408	0.408
YDG708 vs YDG707	α	2	1.393	0.393
YDG708 vs YDG707	α	2	1.679	0.679
YDG708 vs YDG707	α	2	1.313	0.313
YDG708 vs YDG707	α	2	1.510	0.510
YDG710 vs YCS68	a	1	1.190	0.190
YDG710 vs YCS68	a	1	1.132	0.132
YDG710 vs YCS68	a	1	1.079	0.079
YDG710 vs YCS68	a	1	1.144	0.144
YDG710 vs YCS68	a	1	1.138	0.138
YDG710 vs YDG709	a	2	1.238	0.238
YDG710 vs YDG709	a	2	1.239	0.239
YDG710 vs YDG709	a	2	1.226	0.226
YDG710 vs YDG709	a	2	1.336	0.336
YDG710 vs YDG709	a	2	1.215	0.215

Table S1.

Asexual fitnesses of weak signalers and non signalers, relative to wild type signalers. Data used to measure the viability cost of signaling as shown in Figure 2. “Strain” is the strains used for the fitness assay, “Genes deleted” is the number of pheromone genes deleted from the second strain, “Fitness” is asexual fitness of the second strain (the strain with deleted genes) relative to the first strain (the wild type signaler). Genotypes are given in Materials and Methods.

Source of Variation	Sum of Squares	DF	Variance	F	p
Mating Type	0.1454	1	0.1454	17.3751	0.0007
Genes Deleted	0.1178	1	0.1178	14.083	0.0017
Interaction	0.0077	1	0.0077	0.9231	0.3510
Within Samples	0.1338	16	0.0084		

Table S2.

2-Way ANOVA table on viability cost of signaling. Analysis of data in Table S1. There is a significant effect of both the mating type and number of genes deleted on cost of signaling. There is no significant interaction between the two variables. Analysis was done using statistical software R.

Strain	Mating type	Quality	Fitness
YDG710 vs YDG709	a	0.1372	1.0311
YDG710 vs YDG709	a	0.1372	0.9692
YDG710 vs YDG709	a	0.1372	1.0080
YDG710 vs YDG709	a	0.1372	1.0446
YDG710 vs YDG709	a	0.1372	1.0240
YDG710 vs YDG709	a	0.0171	1.1296
YDG710 vs YDG709	a	0.0171	1.1239
YDG710 vs YDG709	a	0.0171	1.0891
YDG710 vs YDG709	a	0.0171	1.1393
YDG710 vs YDG709	a	0.0171	1.1151
YDG710 vs YDG709	a	0.0105	1.0467
YDG710 vs YDG709	a	0.0105	1.1459
YDG710 vs YDG709	a	0.0105	1.1287
YDG710 vs YDG709	a	0.0105	1.1213
YDG710 vs YDG709	a	0.0105	1.0831
YDG710 vs YDG709	a	0.1297	1.0703
YDG710 vs YDG709	a	0.1297	1.0457
YDG710 vs YDG709	a	0.1297	1.0592
YDG710 vs YDG709	a	0.1297	1.0469
YDG710 vs YDG709	a	0.1297	1.0569
YDG710 vs YDG709	a	0.1223	1.0561
YDG710 vs YDG709	a	0.1223	1.0732
YDG710 vs YDG709	a	0.1223	1.0578
YDG710 vs YDG709	a	0.1223	1.0488
YDG710 vs YDG709	a	0.1223	1.0550
YDG710 vs YDG709	a	0.0676	1.0804
YDG710 vs YDG709	a	0.0676	1.0777
YDG710 vs YDG709	a	0.0676	1.0778
YDG710 vs YDG709	a	0.0676	1.0836
YDG710 vs YDG709	a	0.0676	1.0798
YDG710 vs YDG709	a	0.0437	1.0977
YDG710 vs YDG709	a	0.0437	1.0843
YDG710 vs YDG709	a	0.0437	1.0788
YDG710 vs YDG709	a	0.0437	1.0868
YDG710 vs YDG709	a	0.0437	1.0878
YDG710 vs YDG709	a	0.1460	0.9938
YDG710 vs YDG709	a	0.1460	1.0020
YDG710 vs YDG709	a	0.1460	1.0093
YDG710 vs YDG709	a	0.1460	0.9956
YDG710 vs YDG709	a	0.1460	1.0082

YDG708 vs YDG707	α	0.1338	1.0148
YDG708 vs YDG707	α	0.1338	1.0358
YDG708 vs YDG707	α	0.1338	1.0271
YDG708 vs YDG707	α	0.1338	1.0221
YDG708 vs YDG707	α	0.1338	1.0343
YDG708 vs YDG707	α	0.0259	1.1705
YDG708 vs YDG707	α	0.0259	1.1863
YDG708 vs YDG707	α	0.0259	1.1929
YDG708 vs YDG707	α	0.0259	1.1673
YDG708 vs YDG707	α	0.0259	1.1741
YDG708 vs YDG707	α	0.0387	1.1417
YDG708 vs YDG707	α	0.0387	1.1595
YDG708 vs YDG707	α	0.0387	1.1599
YDG708 vs YDG707	α	0.0387	1.1483
YDG708 vs YDG707	α	0.0387	1.1495
YDG708 vs YDG707	α	0.1226	1.0888
YDG708 vs YDG707	α	0.1226	1.0874
YDG708 vs YDG707	α	0.1226	1.0563
YDG708 vs YDG707	α	0.1226	1.0573
YDG708 vs YDG707	α	0.1226	1.0679
YDG708 vs YDG707	α	0.1219	1.0724
YDG708 vs YDG707	α	0.1219	1.0790
YDG708 vs YDG707	α	0.1219	1.0852
YDG708 vs YDG707	α	0.1219	1.0778
YDG708 vs YDG707	α	0.1219	1.0814
YDG708 vs YDG707	α	0.0844	1.0925
YDG708 vs YDG707	α	0.0844	1.0946
YDG708 vs YDG707	α	0.0844	1.0906
YDG708 vs YDG707	α	0.0844	1.0900
YDG708 vs YDG707	α	0.0844	1.0609
YDG708 vs YDG707	α	0.0692	1.1038
YDG708 vs YDG707	α	0.0692	1.1000
YDG708 vs YDG707	α	0.0692	1.1082
YDG708 vs YDG707	α	0.0692	1.1068
YDG708 vs YDG707	α	0.0692	1.1266
YDG708 vs YDG707	α	0.1374	1.0022
YDG708 vs YDG707	α	0.1374	1.0045
YDG708 vs YDG707	α	0.1374	0.9968
YDG708 vs YDG707	α	0.1374	1.0024
YDG708 vs YDG707	α	0.1374	1.0053

Table S3.

The cost of signaling for signalers in different environmental conditions. Data used to generate Figure 3. “Strain” is the strains used for the fitness assay, “Quality” is the quality of the environment determined by the maximal growth rate of a wild type signaler (see Materials and Methods), “Fitness” is asexual fitness of the second strain (the non signaler) relative to the first strain (the wild type signaler). Genotypes are given in Materials and Methods.

Source of Variation	Sum of Squares	DF	Variance	F	p
Quality	0.1377	1	0.1377	286.948	<0.0001
Mating Type	0.0173	1	0.0173	36.051	<0.0001
Interaction	0.0145	1	0.0145	30.14	<0.0001
Residuals	0.0364	76	0.0005		
Total	0.2059	79			

Table S4.

ANCOVA table on cost of signaling for signalers in different environmental conditions. Analysis of data in Table S3 showing a significant effect of environmental quality and mating type on the cost of signaling. There is also a significant interaction between the environmental quality and the mating type. Analysis was done using statistical software R.

Strain	Mating Type	Quality	Fitness
YCS50 vs YCS54	a	0.1338	1.0172
YCS50 vs YCS54	a	0.1338	1.0154
YCS50 vs YCS54	a	0.1338	1.0127
YCS50 vs YCS54	a	0.1338	0.9648
YCS50 vs YCS54	a	0.1338	1.0158
YCS50 vs YCS54	a	0.0259	1.1253
YCS50 vs YCS54	a	0.0259	1.1384
YCS50 vs YCS54	a	0.0259	1.1139
YCS50 vs YCS54	a	0.0259	1.1393
YCS50 vs YCS54	a	0.0259	1.0172

Table S5.

The cost of signaling for signalers in 2 different environments, in genetic background Y55. "Quality" is the quality of environment determined by the maximal growth rate of a wild type signaler (see Materials and Methods), "Fitness" is asexual fitness of a weak signaler (YCS54 *MATα mfα1::KanMX4 ura3*) relative to a wild type signaler (YCS50 *MATα ura3*).

Cost of signaling Strain	Quality	Fitness
YCS50 vs YCS53	1.0000	1.0271
YCS50 vs YCS53	1.0000	1.0659
YCS50 vs YCS53	1.0000	1.0280
YCS50 vs YCS53	1.0000	0.9972
YCS50 vs YCS53	1.0000	1.0216
YCS73 vs YCS75	0.8926	1.0901
YCS73 vs YCS75	0.8926	1.0368
YCS73 vs YCS75	0.8926	1.0549
YCS73 vs YCS75	0.8926	1.0862
YCS73 vs YCS75	0.8926	1.0894
YCS45 vs YCS49	0.7552	1.1239
YCS45 vs YCS49	0.7552	1.0743
YCS45 vs YCS49	0.7552	1.1066
YCS45 vs YCS49	0.7552	1.1474
YCS45 vs YCS49	0.7552	1.1755
YCS176 vs YCS177	0.6846	1.1996
YCS176 vs YCS177	0.6846	1.1951
YCS176 vs YCS177	0.6846	1.1729
YCS176 vs YCS177	0.6846	1.2664
YCS174 vs YCS175	0.5661	1.3519
YCS174 vs YCS175	0.5661	1.3216
YCS174 vs YCS175	0.5661	1.1901
YCS174 vs YCS175	0.5661	1.2652
YCS174 vs YCS175	0.5661	1.3326

Marker control Strain	Quality	Fitness
YCS50 vs YCS64	1.0000	1.0245
YCS50 vs YCS64	1.0000	1.0280
YCS50 vs YCS64	1.0000	1.0100
YCS50 vs YCS64	1.0000	1.0022
YCS50 vs YCS64	1.0000	1.0055
YCS73 vs YCS74	0.8926	1.0119
YCS73 vs YCS74	0.8926	1.0133
YCS73 vs YCS74	0.8926	1.0183
YCS73 vs YCS74	0.8926	1.0087
YCS73 vs YCS74	0.8926	1.0180
YCS45 vs YCS65	0.7552	1.0312
YCS45 vs YCS65	0.7552	1.0131
YCS45 vs YCS65	0.7552	1.0189
YCS45 vs YCS65	0.7552	1.0106
YCS45 vs YCS65	0.7552	1.0017
YCS176 vs YCS179	0.6846	1.0074
YCS176 vs YCS179	0.6846	1.0099
YCS176 vs YCS179	0.6846	1.0078
YCS176 vs YCS179	0.6846	1.0172
YCS176 vs YCS179	0.6846	1.0081
YCS174 vs YCS178	0.5661	1.0208
YCS174 vs YCS178	0.5661	1.0077
YCS174 vs YCS178	0.5661	1.0139
YCS174 vs YCS178	0.5661	1.0065
YCS174 vs YCS178	0.5661	1.0067

Table S6.

The cost of signaling for signalers in different genetic backgrounds. "Strain" is the strains used for the fitness assays, "Quality" is the quality of the genetic background determined by the relative asexual fitness of the first strain (the wild type signaler) relative to wild type signaler YCS50 (*MATα ura3*), "Fitness" is asexual fitness of the second strain relative to the first strain. "Cost of signaling" is the experimental treatment, "Marker control" is a control for the effect of the *URA3* genetic marker. Genotypes are given in Materials and Methods.

Fitness	Cells	Strength
fit	3.8	0.8785
fit	4.4	1.0159
fit	5.0	1.3010
fit	5.8	1.6132
fit	5.6	1.7077
fit	6.0	1.8073
fit	7.5	2.5757
fit	7.0	2.0994
fit	8.9	3.0701
fit	8.5	2.1901
fit	9.0	2.6592
fit	7.0	2.0190
fit	5.5	1.4999
fit	7.0	2.1596
unfit	3.9	0.3203
unfit	5.1	0.5122
unfit	4.9	0.8183
unfit	5.1	0.8209
unfit	5.5	1.1873
unfit	5.9	1.3397
unfit	6.1	1.2650
unfit	5.0	0.5892
unfit	5.3	0.8837
unfit	5.5	0.9754
unfit	6.6	1.1197
unfit	5.7	1.2940
unfit	5.5	1.0352

Table S7.

Effects of genetic fitness on signalling strength (*MATα*).

"Fitness" indicates strain type (high or low quality), "cell" indicates number of cells per colony (x10^{11}), "strength" indicates area of inhibition of tester strain measured (cm^2) as an indicator of signalling strength. Details of protocol given in Materials and Methods.

Fitness	Cells	Strength
fit	4.6	1.2300
fit	5.1	1.2940
fit	5.7	1.5836
fit	6.6	1.6359
fit	6.5	1.8359
fit	6.8	2.0102
fit	6.9	1.9470
fit	7.0	2.2848
fit	7.3	2.4771
unfit	4.9	0.9542
unfit	5.5	0.9849
unfit	5.2	1.0018
unfit	5.8	1.0158
unfit	5.3	1.0817
unfit	5.9	1.1954
unfit	5.4	1.3267
unfit	6.1	1.2803
unfit	6.5	1.4530
unfit	6.6	1.7692

Table S8.

Effects of genetic fitness on signalling strength (*MATa*).

"Fitness" indicates strain type (high or low quality), "cell" indicates number of cells per colony ($x10^{11}$), "strength" indicates area of inhibition of tester strain measured (cm^2) as an indicator of signalling strength. Details of protocol given in Materials and Methods.

i)

Source of Variation	DF	Sum of Squares	Variance	F	p
Fitness	1	6.2677	6.2677	174.2478	<0.0001
Cells	1	5.4621	5.4621	151.8528	<0.0001
Interaction	1	0.0065	0.0065	0.1794	0.6758
Residuals	23	0.036	0.0016		
Total	26	11.7723			

ii)

Source of Variation	DF	Sum of Squares	Variance	F	p
Fitness	1	6.2677	6.2677	180.42	<0.0001
Cells	1	5.4621	5.4621	157.23	<0.0001
Residuals	24	0.8338	0.0347		
Total	26	12.5636			

Table S9.

ANCOVA table on effect of genetic quality on signal strength.

Analysis of data in Table S7. i) shows the analysis including the interaction between the variables. ii) shows the analysis with the interaction removed from the model. Analysis was done using statistical software R.

i)

Source of Variation	DF	Sum of Squares	Variance	F	p
Fitness	1	1.7317	1.7317	62.5490	<0.0001
Cells	1	1.6002	1.6002	57.7977	<0.0001
Interaction	1	0.0028	0.0028	0.0998	0.7564
Residuals	15	0.4153	0.0277		
Total	18	3.7500			

ii)

Source of Variation	DF	Sum of Squares	Variance	F	p
Fitness	1	1.7317	1.7317	66.278	<0.0001
Cells	1	1.6002	1.6002	61.243	<0.0001
Residuals	16	0.4181	0.0261		
Total	18	3.7500			

Table S10.

ANCOVA table on effect of genetic quality on signal strength.

Analysis of data in Table S8. i) shows the analysis including the interaction between the variables. ii) shows the analysis with the interaction removed from the model. Analysis was done using statistical software R.

4. Preference for Mate Size in *Saccharomyces cerevisiae*

4.1 Abstract

The budding yeast *Saccharomyces cerevisiae* is believed to undergo sexual reproduction rarely, between long periods of asexual growth. The fitness of a diploid clone during the long asexual growth phase depends on the genotype of the founding zygote, which is itself determined by the genotypes of its haploid parents. This places strong pressure on haploid cells to evolve mechanisms to discriminate between good and poor quality potential mates when a sexual opportunity arises. A good quality mating can provide important direct benefits, such as a faster growth rate as well as indirect benefits through better adaptation that will pay off during the period of vegetative growth. Here I test the role of cell size as a potentially favourable characteristic. I show that mating with relatively larger spores leads to a doubling rate advantage for the resulting zygote when growth occurs in glucose-rich (optimum) medium. However, on glucose–poor (reduced) medium, mating with a relatively smaller spore is seen to provide a doubling rate advantage. Using mate choice trials, I then show that mate quality discrimination based on cell size is possible during spore-to-spore matings. The difference in germination time between large and small spores varies significantly between optimum and reduced media. This difference may account for the mate quality discrimination as a form of passive selection. My results provide new insight into the as yet still mysterious natural mating behaviour of *S. cerevisiae.*

4.2 Introduction

Being able to distinguish between good and poor mating partners is an important ability for any individual. If an individual makes a poor choice of mate, they run the risk of producing inferior offspring that may never reproduce. A good choice however, will ensure the production of strong healthy progeny, which in turn will proliferate and pass on their genes to future generations. The benefits of a good mate can be either direct or indirect. Direct benefits will be of immediate use to the chooser, and will usually include useful resources such as in the case of nuptial gifts in several insect species (Thornhill 1976), or access to resource rich territories as most commonly studied in birds (Yasukawa & Searcy 1982). Indirect benefits are traditionally described through the good genes hypothesis (Zahavi 1975 & 1977). Here, a good mate has genes that make them better adapted for the current environment. Future progeny will carry these genes, which in turn make them well suited to their environment giving them the opportunity to prosper. So making a good choice in mate ensures higher Darwinian fitness.

Saccharomyces cerevisiae is thought to grow as an asexual diploid most of the time. But when starved of nutrients, diploid cells can undergo meiosis. This produces four haploid spores of two mating types (*MATa* and *MATα*). When these four spores germinate into vegetative cells (Herskowitz 1988), they produce a mate-type specific pheromone to advertise to cells of the opposite mating type. When two cells successfully receive each other's pheromone signal, they can fuse together to form a

diploid (Herskowitz 1988). The period between mating is characterised by several hundred generations of asexual diploid growth.

There are two known mechanisms by which *Saccharomyces cerevisiae* generates non-random mating. The first mechanism is asynchronous germination which is an important prezygotic barrier (Maclean & Greig 2008). Wild populations of *S. cerevisiae* have been found to occupy the same niche as their closest relative *Saccharomyces paradoxus* in deciduous woodland (Naumov et al 1998). Asynchronous germination between the two species means that they do not mate at the same time. This prevents costly matings between the two species. When these two species mate, they produce infertile offspring, so it is important to avoid inter species mating. As they germinate at different rates, each species only gets the chance to mate with one of its own.

The second mechanism occurs when cells have multiple potential mates to choose between. Cells of both mating types are attracted to the strongest source of pheromone released by cells of the opposite mating type (Jackson & Hartwell 1990). A recent study showed that the pheromone is not only costly to produce, but that this cost is condition-dependent with respect to cell quality, such that for good quality cells the cost is less and so produce more pheromone (Smith & Greig 2010). Unlike the passive discrimination attributed to germination times in the first mechanism (Maclean & Greig 2008), this is a case of true mate choice through honest signalling, and allows cells to mate with the best quality partner available offering indirect benefits as per the good genes theory (Zahavi 1975 & 1977). However, due to the lack of studies into the

ecology of yeast, it is difficult to see a practical application of this honest signalling under natural conditions. Without knowing how, when or why yeast mate we do not know in what context they are signalling to each other. But given the high cost of producing the pheromone (Smith & Greig 2010) and that more pheromone is produced than is required for mating (Hagen & Sprague 1984, Hartig et al 1986, Jenness & Spatrick 1986, Strazdis & MacKay, 1983, Achestetter 1989), it seems unlikely that such a system would evolve without a very useful purpose.

The two mechanisms of non-random mating in *S. cerevisiae* are likely to yield both direct and indirect benefits. If the founding cell of a colony grows faster than its competitors, it will gain primary access to nutrients and space as it undergoes multiple rounds of mitotic growth. In addition, indirect benefits are possible. Due to the large number of asexual generations between sexual reproduction events, there is likely to be a build of deleterious mutations in lineages that reproduce asexually (Muller 1964). These potentially can be purged through sex and recombination (Kondrashov 1988). As a result of both of these potential benefits, there is likely to be strong selection for discrimination between good quality and poor quality potential mates.

Spore size could be a character that is associated with fitness benefits. A larger mating partner provides more cytoplasmic material, and helps the resulting diploid cell reach the critical size for the START phase of the cell cycle faster (Johnston et al 1977), and so bud before its competitors. In contrast, in an environment where nutrients are a limiting factor, a smaller cell might be more efficient than a larger competitor, as it

will have a more favourable surface area per unit volume ratio. When nutrients are a limiting factor, efficiency becomes much more important than when nutrients are in abundance. A good choice in mate size should results in earlier production of a meiotic daughter cell from the resulting zygote. By growing faster than it's competitors, the new colony will have primary access to nutrients as well as space. If spore size is indeed a character that is associated with fitness benefits does *S. cerevisiae* display a preference for mate size? If such a preference does exist, is the discrimination due to honest signalling through mating pheromone, or is it due to asynchronous germination times?

Here I test the hypothesis that mating with a larger cell is beneficial by taking measurements of initial growth rate of small and large diploid cells. Having established that it is advantageous to discriminate between different sized potential mates, I then show that *Saccharomyces cerevisiae* display a clear preference when given a choice of size in mating partner. I then attempt to explain how mate size is discriminated for during the mating process.

4.3 Methods

4.3.1 Strains and media

Two strains were used derived from the common *Saccharomyces cerevisiae* laboratory strain Y55 (McCusker & Haber 1988) . YCS59 *MATa/α ho/ho ura3/ura3 arg1/arg1* and YCS60 *MATa/α ho/ho lys2/lys2 his4/his4*. The auxotrophic markers allowed either strain to be identified

using drop out media. A hybrid of the two strains will grow on minimal media as a result of the complementation of the markers.

To produce large spores, strains were plated on potassium acetate medium (2% potassium acetate). To produce small spores a lower concentration of potassium acetate was used (0.01% potassium acetate). During the sporulation period, strains were incubated at 25°C for 7 days as per standard laboratory protocol(Burke et al 2000). The result of meiotic division is the tetrad ascospore, made up of four haploid spores. The incubation period assured a high number of tetrads per strain. Mate choice assays and observations were done both on rich (YEPD, 2% glucose) and poor (YEPD-, 0.2% glucose) media and incubated at 30°C for optimal vegetative growth .

4.3.2 Rich medium:

4.3.2.1 Measures of initial growth of zygotes

To measure the effect of spore size on initial growth, zygotes of either two large or two small spores were produced using a Zeiss micromanipulator microscope. In each case the two spores used were from different strains, such that both the large and small diploids had the same genotype with respect to auxotrophic markers. After a successful mating, each zygote was observed under the microscope and timed for one complete generation of asexual growth to give a measure of the initial growth rate.

4.3.2.2 Mate choice assays

To test for preferential mating, mate choice assays were done using a previously developed technique (Maclean & Greig 2008). Each trial consisted of three spores. Strains were treated using standard laboratory zymolyase protocol (Burke et al 2008) to digest the ascus, giving access to the spores. Two spores from one strain/size where isolated from separate tetrads. These two spores were then put into contact with a third spore taken from the other strain/size on a plate of rich medium. There were 8 possible combinations that may have resulted from arranging spores in this way. As shown in Fig. 1., mate choice can only occur when the two cells of the same size/strain were of different mating types. One of those two spores will have had a choice of mating with either the other spore from its own strain/size, or the spore of the other strain/size. Plates were then incubated for 4 hours. This time allowed spore germination and mating to take place. Upon checking of plates, any unmated individuals were removed and placed on an unused part of the plate, leaving the zygote to grow as a pure colony. If the unmated spore failed to produce a colony it was deemed dead, and the test was not included in the results as no choice was possible.

As the two strains being used had different markers, the assay was repeated using 4 combinations of size and strain to test for any marker effects. These combinations were:

	YCS59	YCS60
i)	2 large spores	1 small spore
ii)	1 small spore	2 large spores
iii)	2 small spores	1 large spore
iv)	1 large spore	2 small spores

4.3.2.3 Analysis of mate choice assays

It is impossible to distinguish between *MATa* and *MATα* when spores are arranged in this way. There were 8 possible combinations of mating type of the 3 spores (Fig. 1.). From these 8, only 4 offer a choice in mate (informative matings). In these instances, there is a spore of each mating type from the chooser strain, giving the chooser strain the possibility of mating with a spore from the same strain as itself or with one from the other strain. The remaining 4 possible combinations do not result in mate choice (uninformative matings), as both spores from the chooser strain are be of the same mating type, with the spore from the other strain being of the opposite (resulting in a mating between strains) or of the same mating type (resulting in no possible matings). If mating is random, 2/3 of all identified matings will result from YCS59 mating with YCS60 (1/3 from forced matings where no choice is present, and 1/2 of the remaining 2/3 where choice is possible [1/3 + (1/2 x 1/3) = 2/3]. Deviation from the proportions of matings expected under conditions of random mating was calculated using a χ^2 test (see Figure 1 for the 8 possible combinations).

The measure of preference for large spores was calculated as the proportion of matings with large spores from the total number of mated trials where this was possible. Where large spores were given the choice, 1/3 of the trials will only have offered an uninformative mating between a large and a small spore. Therefore, preference = total number of matings between two large spores/ [(2/3)*total number of observed matings]. Where small spores were given the choice of mating partner, mating with

a large spore was always an option. Here preference = total number of matings between a small and large spore/ total number of observed matings. Both formulas give a number from 0-1. A value of greater than 0.5 indicates preference for large spores, a value of smaller than 0.5 indicates preference for small spores.

4.3.2.4 Germination times

To estimate germination time, spores were observed until they began to bud. This was done by plating individual spores on plates of medium. These were then incubated at 30°C for 4 hours. After this, the spores were check at regular intervals for signs of budding.

4.3.3 Poor medium:

4.3.3.1 Measures of initial growth of zygotes

Measures of initial growth of zygotes were done in the same way as on rich medium.

4.3.3.2 Mate choice assays

The assay was performed in the same way as on rich media. Here the assay was only done using strain YCS59 as the chooser strain. It was established on rich media that both strains behaved in the same way regardless of which role they were used in. The combinations used for the assay were:

	YCS59	YCS60
i)	2 large spores	1 small spore
ii)	2 small spores	1 large spore

4.3.3.3 Germination times

Estimates of germination time were measured in the same way as on rich medium. Here, the initial incubation period at 30°C was for 6 hours, to allow for the slower growth rate on the poor medium.

4.4 Results

4.4.1 Rich Environment:

4.4.1.1 Measures of initial growth of zygotes

Zygotes, produced from the successful mating of two large spores, produced independent daughter cells after a mean time of 82.12 minutes (n=17, standard deviation =2.472). Zygotes, produced from two small spores, produced independent daughter cells after a mean time of 113.55 minutes (n=11, standard deviation =14.528). This difference of roughly 30 minutes indicates that larger cells had a significant growth advantage over small cells on rich media ($t=7.11$, $DF=10$, $p<0.001$).

4.4.1.2 Mate choice assays

On the rich medium, large spores were preferred as mates significantly more often than small spores (preference for large spores on rich medium = 0.803 $\chi^2=36$, $DF=1$, $p<0.001$). When large spores were given a choice of mating with a large or small spore on rich medium, they chose to mate with the other large spore more often than expected by

chance (χ^2=13.885, DF=1, p<0.001). This was observed for both combinations of strain size and marker (YCS59 large spores: χ^2= 6.896, DF=1, p=0.008; YCS60 large spores χ^2=7.000, DF=1, p=0.006) (Figure 2 – pooled data). This resulted in a preference of 0.74 for large spores choosing to mate with other large spores.

When small spores were given the choice of mating with a large or small partner, large spores were still chosen more often than expected (χ^2=16.178, DF=1, p<0.001). This choice was significant for both combinations of strain size and marker (YCS59 small spores: χ^2=7.511, DF=1, p=0.006; YCS60 small spores: χ^2= 8.675, DF=1, p=0.013) (Figure 2). This resulted in a preference of 0.84 for small spores choosing to mate with large spores. Although both spore sizes displayed significant preference for large spores, small spores had a significantly stronger preference than large spores (χ^2=6.027, DF=1, p=0.014).

4.4.1.3 Germination times

The time taken from plating of a spore, to initial bud formation was measured for both large and small spores and used as a measure of germination time. Large spores showed initial bud formation after a mean time of 299 minutes (n=20, standard deviation =9.403). Small spores showed initial bud formation a mean time of 336.75 minutes (n=20, standard deviation =7.656). These results suggest that large spores germinate significantly faster than small spores on rich media (t=13.92, DF=38, p<0.001).

4.4.2 Poor Environment

4.4.2.1 Measures of initial growth of zygotes

On poor media, large zygotes produced independent daughter cells after a mean time of 570.8 minutes (n=5, standard deviation =73.183). Small zygotes produced independent daughter cells after a mean time of 350 minutes (n=5, standard deviation =108.630). This indicates a significant advantage for small cells on poor medium (t=3.77, DF=8, p=0.005).

4.4.2.2 Mate choice assays

Here, small spores were preferred as mates more often than small spores (preference for large spores on rich medium = 0.404). When large spores were given a choice of mating with a large or small spore, they chose to mate with small spores more often than expected by chance (χ^2=8.261, DF=1, p=0.004). This represented a preference of 0.242 for large spores choosing to mate with other large spores.

When small spores were given the choice of mating with a large or small partner, small spores were still chosen more often than expected by chance (χ^2=4.716, DF=1, p<0.001. Although this resulted in preference for large spores of 0.462 it was not small enough to be deemed significant (χ^2=0.640, DF=1, p=0.424). This suggests that small spores have no preference with respect to spore size when mating. This is a significant difference to the preference for large spores seen when large spores were given a choice of mate size (χ^2=19.485, DF=1, p=0<0001) (Fig. 2.).

4.4.2.3 Germination times

Small spores showed signs of early bud formation faster than large spores. Small spores took a mean time of 401.5 minutes (n=20, standard deviation =15.600), while large spores took a mean time of 617.25 minutes (n=20, standard deviation =11.821). This is a significant difference (t=49.3, DF=35, $p<0.001$) in germination time for small spores on poor medium.

4.4.3 Comparison of mate size preference on rich and poor media

In a rich environment, large spores were preferred as mates (preference for large spores = 0.803), however in the poor environment preference for large spores fell bellow 0.5 (preference for large spores = 0.404) signifying a preference for small spores (preference for small spores = 1-0.404 = 0.596). When considering overall preference for desired mate size, preference was far stronger on the rich environment (χ^2=16.667, DF=1, $p<0.0001$). This result is misleading as large cells display similar levels of preference for their desired mate size in both environments. On the rich environment, large spores had a preference of 0.74 for large spores. In the poor environment, they had a preference of 0.76 for small spores. Large spores displayed constant levels of preference in both environments (χ^2=0.29, DF=1, p=0.640). Small spores on the other hand behaved very differently in the two environments. On the rich environment they had a preference of 0.84 for large spores. However on the poor environment they had a much weaker preference of

0.54 for small spores. This is a very significant difference strength of preference (χ^2=36.232, DF=1, p<0.0001).

4.5 Discussion

4.5.1 Benefits of size reflected in mate preference.

The results show that initial zygote fitness is determined by the size of the parental spores, with zygotes derived from large spores being better on glucose-rich media and zygotes derived from small spores being better on glucose poor environment. This suggests that mate choice for size should evolve, and indeed we find that large mates are preferred on rich medium and small mates are preferred on poor medium.

To be more specific, in the rich environment, large zygotes had higher growth rates and budded daughter cells faster than small zygotes. If a large zygote and a small zygote produced competing colonies, this half hour head start for the large zygotes would represent a significant advantage. The growing colony would have primary access to nutrients and available space, so it will likely be more populated than the competing colony trailing behind. In contrast, in the poor environment, small zygotes had higher growth rates and budded daughter cells faster than large zygotes. So the fitness advantage switches to smaller cells in environments with restricted access to resources.

These results show that spore size is clearly a trait worth discriminating by a mating partner. The mate choice assays suggest that discrimination based on size does in fact take place. Deviations from the mating ratios expected under random mating were calculated using χ^2

tests (see methods and Fig. 1.). In each case it was established that mating partners were being selected non-randomly, with discrimination on their size. In the rich environment, large spores were preferred, and in poor environments small spores were preferred.

Although there was a general preference for size in both environments, cells behaved differently under the two conditions. When pooling results, preference was much stronger in the rich environment. However, this difference in perceived preference is due mainly to differing behaviour of the small spores. Where large spores displayed similar levels of preference in both rich and poor environments, small spores displayed a relatively strong preference for large spores in the rich environment, but a negligible preference in the poor environment. So in the rich environment, small spores displayed a stronger preference for mate size, but in the poor environment large spores displayed a stronger preference.

4.5.2 Mechanism for preference

On the rich medium, large spores began to bud faster than small spores, and on the poor medium, small spores began to bud faster. This result is relevant to the observed mate discrimination. Spores that germinate faster will be ready to mate faster. So when large spores were given a choice of mate on the rich medium, it is possible that the observed preference was simply due to the two large spores being ready to mate before the small spore. Similarly, when small spores were given the choice of mate on the poor medium, they will have been ready to mate

earlier than the big spore, accounting for their observed tendency to mate with other small spores.

Readiness to mate, may also account for the observed mating preferences in the other two trials. When small spores where given choice on rich medium, they also preferred to mate with large spores. Both small spore will have germinated more slowly than the large spore, and have been exposed to a mature large spore already signalling its readiness to mate. So the large spore would gain a mating advantage. The same logic applies to the symmetrical situation on poor medium, but now the single small spore germinated more quickly and gained a higher chance of mating.

Differences in germination time can also explain the difference in preferences seen between chooser strain size. Fig. 2. shows that preference for the most desirable mate is stronger in cases where the undesirable mate is given the choice. On the rich medium, small spores displayed a larger preference for large spores, whereas on the poor medium, large spores displayed a larger preference for small spores than that seen in small spores. I hypothesize that on the rich media, when small spores were given the choice of mating partner, the large cell will have been signalling on its own while the small spores were germinating. This extended period of time will have made the large cells very attractive due to the amount of mating pheromone they will have produced. However, when the large spores were given a choice they will both germinate at approximately the same rate. At this stage, neither cell is particularly attractive, as they will not have been producing mating

pheromone for very long. The majority of the time, the two large cells will still mate. However, if they fail to mate one cell may start to under go mitosis. At this point it will be unable to mate. This would leave the remaining large cell as a very attractive partner available for the newly germinated small cell to mate with. The reverse would be true on the poor medium, where small spores mature faster than the large spores. To test this hypothesis, the mate choice trials could be repeated taking germination times into account. Plating the slower germinating spores first so that all 3 spores are ready to mate at the same time.

In *Saccharomyces cerevisiae,* cells need to reach a critical size before budding. This critical size is determined by the growth rate of the cell, which in turn is affected in this case by the concentration of glucose in the medium (Thievelein 1994). In the glucose rich environment, the growth rate was faster than on the poor medium. In the rich environment, large cells will be closer to the critical budding size than small cells, so will have to grow for a shorter period of time than the small cells before producing daughter cells. Small cells on the other hand need to grow for a longer period of time until they reach this critical size, only then can they begin to produce daughter cells. This accounts for why large zygotes produced daughter cells faster than small zygotes on rich media. This works because of the abundance of glucose in this environment, so the big cell is never in short supply. On the poor medium, glucose was scarce. Large cells were at a disadvantage here. Although they still had a large surface area, the ratio of surface area to cell volume is far from favourable. Unbudded cells are spherical (Herskowitz 1988) so will have

a surface are of $4 \pi r^2$ and a volume of $4/3 \pi r^3$. This gives a volume to surface area ratio of $r/3 : 1$ where r is equal to the radius of the cell. As r increases, the ratio becomes less favourable with respect to cell efficiency. So when glucose is scarce as in the poor medium used in these experiments, the small cells will have been much more efficient and therefore grew faster. As the growth rate on this medium was far slower, the critical size needing to be reached before producing daughter cells will have been much smaller here. It is possible that in this case, the small spores were closer to the critical size, so there was a reversal of roles from the experiments on the rich medium. Here the large spores may have faced a lengthier period of time than the small cells reaching the critical cell size for the environment, before being able to produce daughter cells.

The differences in these ratios are important when considering the steps required for spore germination. Glucose is a crucial first step in the Ras-adenylate cyclase pathway that commits a cell to germinate (Thievelein 1994). In the glucose rich environment, large spores are able to take in as much as they need due to its abundance in the medium. During the process of sporulation cells need to grow, so here the large spore is at an advantage over the small spore. However, in the poor medium that is extremely low in glucose, it is the small spores that are more efficient at taking in glucose to begin the process of germination faster than the large spores.

4.5.3 Relationship between sporulation condition and growth conditions

Large spores were produced faster than small spores. Although both were incubated for the same amount of time (7 days), large spores usually formed after about 2-3 days. This corresponds with the slower growth rates seen on poor medium on which smaller spores were better suited, and the faster growth rates on rich media on which large spores were better suited. It would be interesting to determine whether the sporulation condition is a precursor for future vegetative growth environment in wild populations. If there were no obvious relation between sporulation conditions and future vegetative growth conditions, a different strategy would have to be used by wild strains, otherwise they would be at high risk of producing poorly adapted spores. As we know that sex takes place rarely, this would seem a big disadvantage to gamble with. Perhaps then, when future vegetative growth conditions constantly vary, diploid cells may produce tetrads of non-uniform sized spores. This would ensure that at least one spore might be well suited to the richness of the new environment.

4.5.4 Conclusion

I show that parental spore size can determine offspring fitness, and that mating cells chose partners on the basis of their size in order to maximise offspring fitness. Large spores are advantageous and preferred in the rich medium, and small spores are advantageous and preferred in the poor medium. An advantageous mating results in a faster initial

growth rate for the zygote. Differences in germination times, allow for this desirable benefit to be discriminated for.

4.6 References

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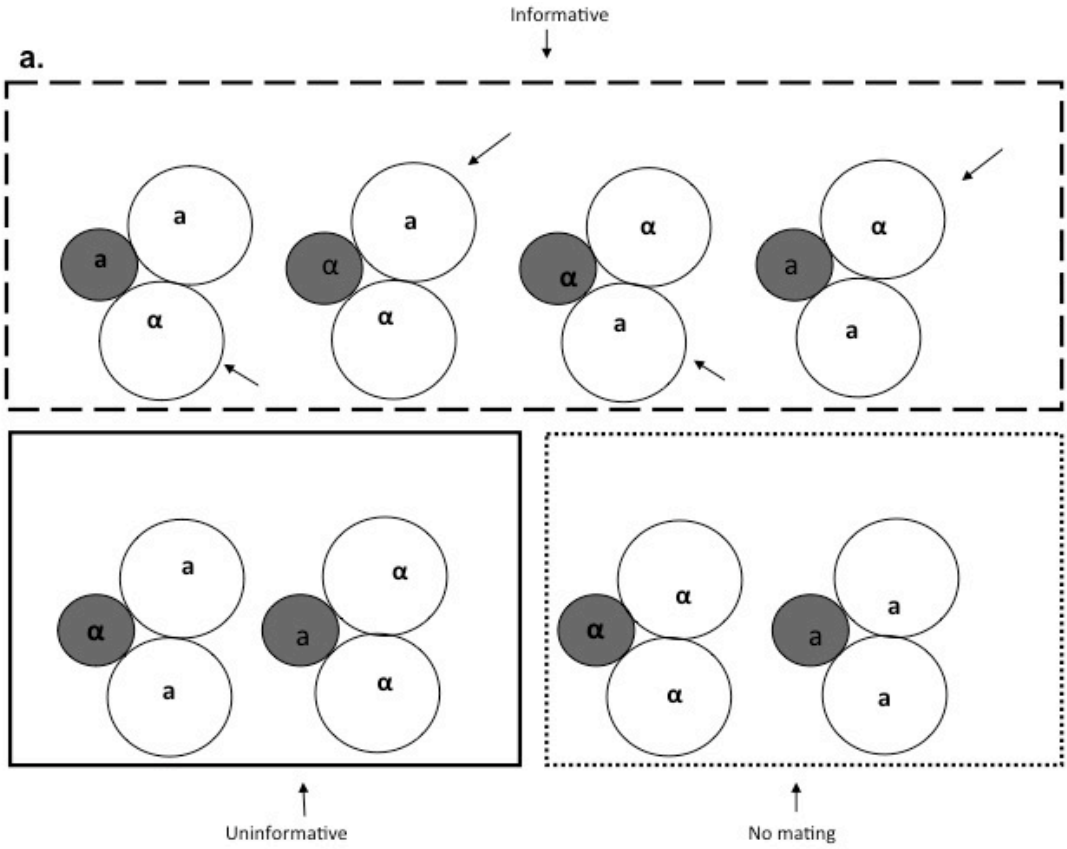
4. 7 Figures


Informative
a.
Uninformative
No mating

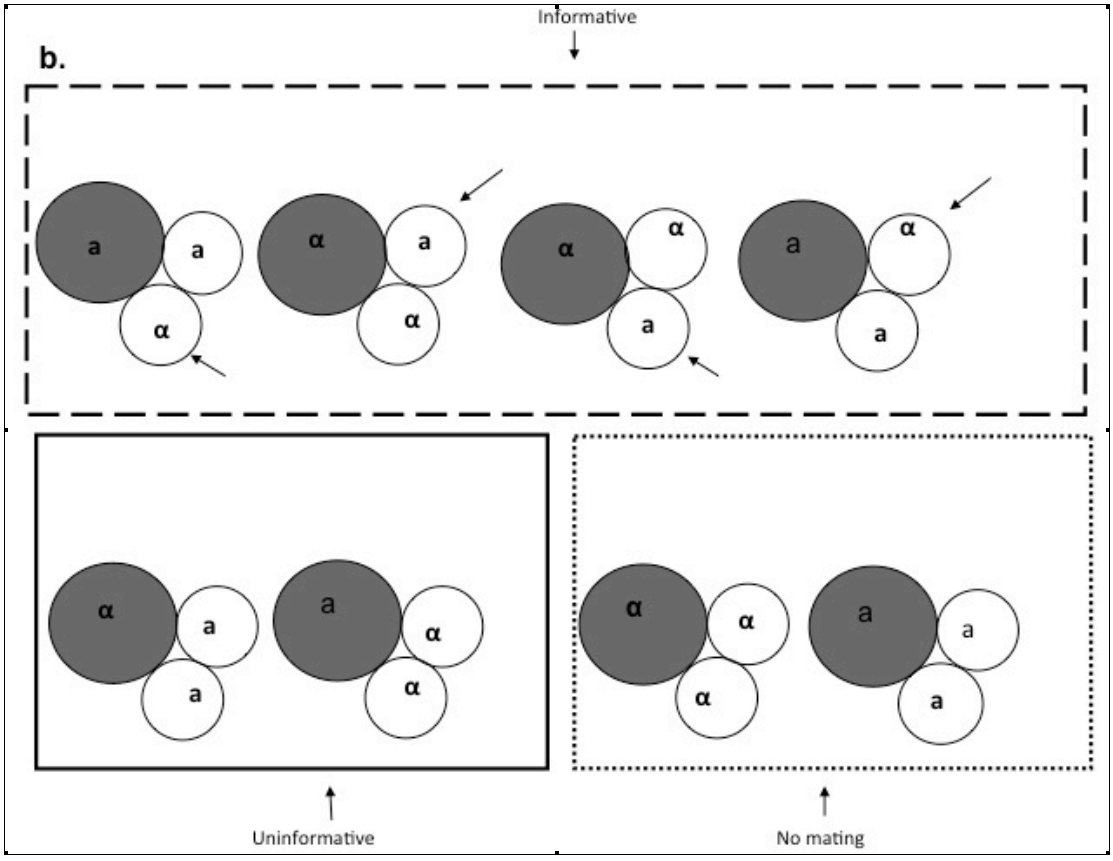

Informative
b.
Uninformative
No mating

Figure 1.

8 possible spore combinations in the mate choice assay. Dark circles represent cells of one strain, white circles represent cells of the other strain. 'a' represents a *MATa* spore, 'α' represents a *MATα* spore. Figure a, shows the assay where large spores are give a choice of mating partner. Figure b shows the assay where small spores are given a choice of mating partner. Only half of the 8 combinations actually offer a choice in mate (the spore with the choice is marked with an arrow in the 'informative' box). Two of the combinations will result in no choice in mating partner between the two strains (Uninformative), while the final two combinations offer no mating (No Mating). If mates are selected randomly, 2/3 of the total matings are expected to be between small and large spores. This includes 1/3 where no choice in mating partner was present, and half of the 2/3 where choice was present. Deviation from this expected value determined whether preferential mating took place with using a χ^2 test.

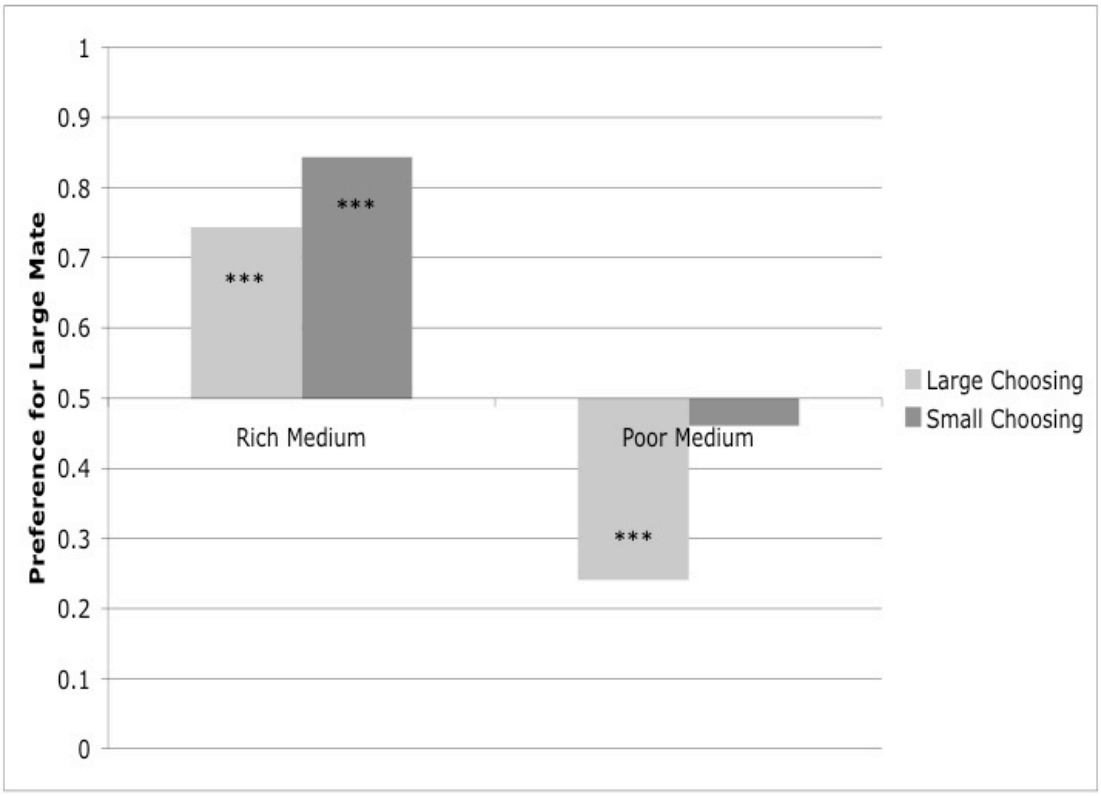


Figure 2.

Preference for mating with larger cells. The graph shows a clear preference for mating with larger cells when mating took place on the rich medium. In contrast however, preference for larger cells fell below 0.50 on the poor medium indicating a preference for smaller cells on this glucose limited medium. The measure of preference was calculated as the proportion of matings with large spores from the total number of mated trials where this was possible. Where large spores were given the choice, 1/3 of the trials will only have offered a mating between a large and a small spore. In these cases, preference = total number of matings between two large spores/ [(2/3)*total number of observed matings]. Where small spores were given the choice of mating partner, mating with a large spore was always an option. Here preference = total number of matings between a small and large spore/ total number of

observed matings. Both formulas give a number from 0-1. A value greater than 0.5 indicates preference for large spores, a value smaller than 0.5 indicates preference for small spores.

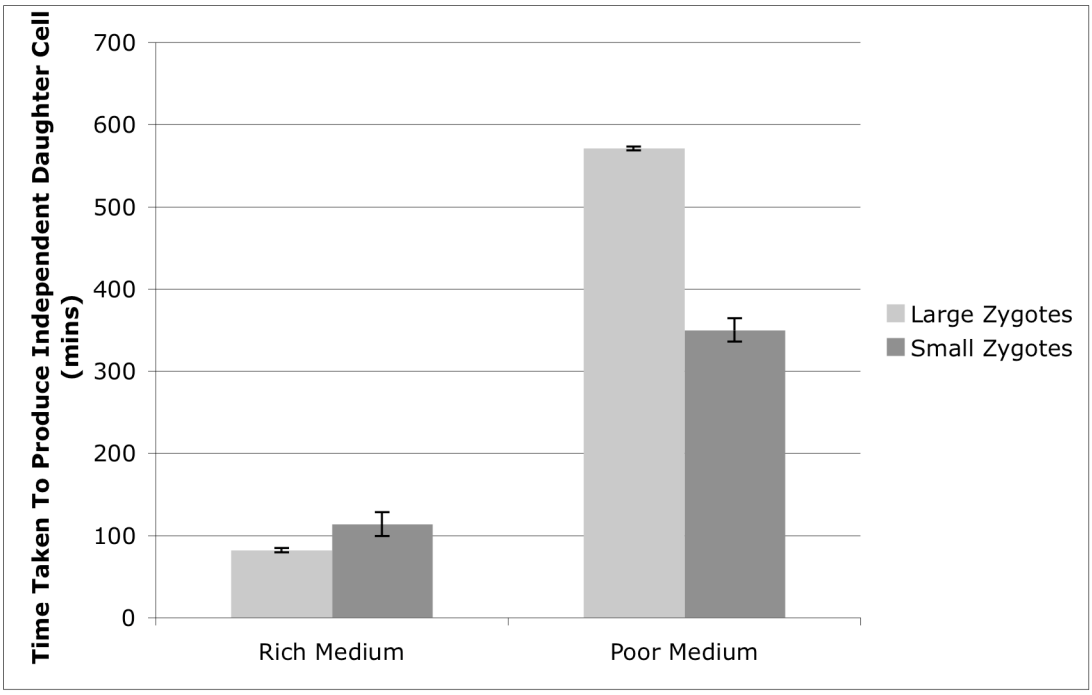


Figure 3.

Difference in doubling time for large and small zygotes on the rich medium and the poor medium. Large zygotes were produced by mating two large spores. Small zygotes were produced by mating two small spores. These were then timed for the production of independent daughter cells. A faster division time is observed for larger zygotes on the rich medium, however on the poor environment it is the smaller zygotes which show a faster relative doubling time.

4.8 Tables

Medium	Chooser Strain	Chooser Strain Size	Total Trials	Total Matings	Obs.Matings With Big Cell	Expected Matings With Big Cell	Preference for Big Cells
Rich	Pooled	Big	239	117	58	39.00	0.744
Rich	YCS59	Big	120	61	30	20.33	0.738
Rich	YCS60	Big	119	56	28	18.67	0.750
Rich	Pooled	Small	237	115	97	76.67	0.643
Rich	YCS59	Small	117	56	47	37.33	0.839
Rich	YCS60	Small	120	59	50	39.33	0.847
Poor	YCS59	Big	120	62	10	20.67	0.242
Poor	YCS59	Small	113	65	30	43.33	0.462

Table 1.

Large cells are more attractive than small cells on rich medium, and small cells are more attractive on poor medium.

Expected number of inter-strain matings was calculated as 2/3 of total matings (see methods). Each trial consisted of 3 spores. The designated chooser strain provided 2 of the 3 spores per trial. Preference was measured as the proportion of total large cell matings. When the large cells were given the choice, the option for the two large cells to mate with each other was only present 2/3 of time time (Fig.1.). So here preference = total matings with large cell/[(2/3)*total matings]. When the choosing strain was small, it was always possible to mate with a large cell. Here, preference = total matings with large cell/ total matings.

Pooled data is of the two assays of the corresponding chooser strain size.

5. Preferential Mating Observed in Intra-tetrad Matings of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*

5.1 Abstract

When *Saccharomyces* yeast goes through meiosis, the products are four haploid spores encased in an ascus forming a tetrad. It is thought that these four gametes will most commonly mate with each other. Each cell signals its availability to mate by secreting a mate-type specific mating pheromone. This pheromone has been shown to be costly to produce, such that higher quality cells can produce a stronger signal compared to lower quality cells. As haploid cells are attracted to the strongest signaller it is thought that preferential mating may be taking place within the tetrad, helping to purge deleterious mutations from the population. This theory has already been shown to be a plausible strategy for *Saccharomyces* yeast to adopt through mathematical modelling. Here I test for preferential mating within the tetrad experimentally in the yeast species *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*. Through mate choice assays I show that homozygous matings are preferred in both species when meiosis produces two high quality spores and two low quality spores. Potential mating barriers are also investigated after low observed mating propensity in the mate choice trials. These results show that purging of deleterious mutations through homozygous matings takes place in yeast, and also give some insight into the function of intra-tetrad mating.

5.2 Introduction

Sexual selection results in adaptations that increase mating success, a specific component of fitness, often at a cost to other components of fitness. Such adaptations include traits to defeat competitors of the same sex and traits to attract mates of the other sex (Andersson 1994). The difference in the traits that evolve under these two modes of sexual selection have been well studied in many systems (Andersson 1994).

A key question that remains unresolved is why are mates attracted by particular traits? In some cases, a trait can convey a direct benefit. This can come in the form of gifts or access to resources. For example in many insect species males present females with gifts at mating (Thornhill & Alcock 1983) that in some cases can increase female fecundity (Thornhill 1976, Gwynne 1984) or give their offspring a greater chance of survival (Dussourd et al 1988). In some bird species such as *Agelaius phoeniceus* (red-winged blackbird), males are chosen as mates based on the quality of their territory (Williams 1975, Emlen & Oring 1977). However, in some examples, females do not obviously gain a direct benefit and may be gaining indirect genetic benefits. 'Good genes' models predict that well ornamented males produce the most healthy offspring. The handicap principle explains this by suggesting a cost to sexual displays, whereby the best quality males can suffer the cost of a larger display (Zahavi 1975 & 1977). Therefore, if males of better genetic quality produce larger sexual displays, then females that are attracted to the displays will produce offspring that inherit good genes from the attractive males.

Sexual selection results from competition for mates. Sexual selection is usually studied in sexually dimorphic species in which males have conspicuous traits, such as fish, stalk-eyed flies and swallows (Andersson 1994, Maynard Smith & Harper 2003). However, sexual selection occurs whenever there is competition for mates, and so can even occur in a selfing hermaphrodite like *Saccharomyces* yeast. Using *Saccharomyces* to study sexual selection offers many advantages over higher organisms. A generation time of just a few hours makes long-term experiments much more feasible. Several yeast species also have fully sequenced genomes. This coupled with established laboratory protocols make *Saccharomyces* very easy to manipulate at a genetic level rather than having to crudely alter characteristics (e.g. sticking extra feathers on swallows).

Much of yeast ecology remains enigmatic, but one well documented fact is that colonial growth takes place mitotically, with individuals reproducing asexually (Herskowitz 1988). However, when it is starved of nitrogen, *Saccharomyces* will sporulate (Phaff et al 1966). During sporulation, a diploid cell undergoes meiosis. This results in a tetrad of haploid spores that are held together by an outer casing called an ascus. Unlike diploid cells, haploid cells have two different sexes (Herskowitz 1988). These are classified as mating type '**a**' and '**α**'. In a tetrad of spores, there will usually be two *MAT****α*** and two *MAT****a.*** Spores are resistant and can remain dormant for a long time. Relative to vegetative cells, spores are much more resilient, withstanding freezing, drying, extreme temperatures and even harmful chemicals (Phaff et al

1966). The spore wall is very different to the vegetative cell wall. One of the characteristics that make it so resilient is a superficial lipid layer that is not present in vegetative cell walls (Miller & Hoffmann-Ostenhof 1964). As a result of their environment, spores metabolise differently to vegetative cells. The cell content of spores is also very different, and contains many more carbohydrates but far fewer free amino acids. Spores need nitrogen and glucose to germinate back into vegetative cells. Glucose catabolites repress enzyme synthesising systems in the cell, returning it to vegetative metabolism. When spores return to a vegetative state, haploids of opposite mating types will mate by fusing together to form a diploid. Current literature supports that the majority of spores mate within their tetrad and consequently each sporulated diploid produces two diploid zygotes (Tsai et al 2008). The assumed high levels of intra-tetrad mating is now disputed in light of recent experimental data (Murphy & Zeyl 2010).

Mating in the tetrad could increase the chance of resulting offspring being affected by recessive deleterious mutations. So inbreeding might be expected to be actively discouraged. Outbreeding strategies offer considerable benefits in increased genetic variation. This could be instrumental in bringing together independently occurring beneficial mutations (Crow & Kimura 1965). So why have *Saccharomyces* evolved a mating system that would seem to promote homozygosity over heterozygosity?

As different species of *Saccharomyces* are able to mate with each other, one hypothesis could be that promoting inbreeding reduces the

chance of producing sterile hybrid offspring. There are mechanisms in place that, while they do not eliminate hybrid matings, do reduce their frequency substantially. These mechanisms are typically observed as species germinating at different rates, so that they tend not to mate at the same time (Maclean & Greig 2008). Some species also display a higher affinity for their own species' mating pheromone (Marsh & Herskowitz 1988). As none of these prezygotic barriers are perfect, it is possible that intratetrad mating has evolved to help keep species apart. However there is an important aspect of yeast mating that may help explain things further.

Haploid cells each produce a mate type specific pheromone. This attracts potential mates of the opposite mating type. When there is choice, cells will chose to mate with whichever cell is producing the most pheromone (Jackson & Hartwell 1990). The *Saccharomyces* mating pheromone can be characterised as an honest signal under the 'handicap principle' (Zahavi 1975 & 1977). The expression of the pheromone is dependent on the individual's phenotypic quality. It is costly to produce and maintain. For individuals in good phenotypic condition, the cost of increased pheromone production is smaller than for individuals in poor phenotypic condition (Smith & Greig 2010). While most sexual displays evolve to improve an individual's chances of mating, this seems an unlikely explanation for the pheromone signalling in *Saccharomyces*. With matings taking place within the tetrad, each cell is guaranteed a mate, as there are two *MAT*$\boldsymbol{\alpha}$ cells and two *MAT*$\boldsymbol{a}$ cells. Even if a spore dies, leaving a cell without a partner to mate with, the unmated haploid can still

produce a daughter cell, then switch mating type and mate with it. *Saccharomyces* therefore have a relatively easy time finding a mate. So the pheromone may not improve the chances of finding a mate, but it may help find the best mate. *Saccharomyces* are attracted to the strongest source of pheromone. This suggests that within the tetrad, the two cells producing the most pheromone will mate with each other, while the two weakest producers will mate with each other. As pheromone strength is an honest signal, this means the cells that produce the most pheromone will be the highest quality cells. Being haploid cells, their phenotypic quality will accurately represent their genetic quality. So, this preferential mating in the tetrad should result in one zygote from a high quality mating and one zygote from a lower quality mating.

The advantage of such preferential mating becomes more apparent when we consider the predominantly asexual nature of *Saccharomyces*. Due to recurrent mutation, an asexual lineage will tend to accumulate deleterious mutations through time, even though selection will tend to mitigate this, favouring those members of a lineage with fewer deleterious alleles. The build up in part occurs due to the generally recessive nature of deleterious mutations (Muller 1964, Lynch & Gabriel 1990). Such a build up can be counteracted in *Saccharomyces* by rare opportunities for sexual reproduction. Due to recombination and independent assortment of loci during meiosis, spores within the tetrad will be genetically different to each other. This means the four spores will be of different relative quality to each other. Those of higher quality will have fewer deleterious mutations. This will allow them to produce more

mating pheromone than the lower quality cells which will have the most harmful deleterious mutations. A mating of two high quality cells will produce a high quality diploid, while two low quality cells will usually produce a lower quality zygote homozygous for harmful mutations. As the higher quality diploid would outcompete the lower quality diploid, the homozygous deleterious mutations in the lower quality diploid would be eliminated from the population. This process of eliminating deleterious mutations from the population was first presented as the 'deterministic mutation hypothesis' (Kondrashov 1988). This idea, of purging deleterious mutations through rare occasions of selective sexual reproduction, is theoretically a viable strategy in yeast (Tazzyman et al in preparation) and provides a very good explanation for the costly signalling system found in *Saccharomyces cerevisiae*.

The experiments presented here were carried out in both *Saccharomyces cerevisiae* and its closest relative *Saccharomyces paradoxus*. *S. cerevisiae* has been associated with human domestication for quite some time, whereas *S. paradoxus* remains a predominantly wild species of yeast. These different evolutionary histories may mean that the two species have been under different selective pressures, leading to different characteristics. Genomic data from two populations of *S. paradoxus* estimated that mating within the tetrad took place 94% of the time, mating due to mate type switching to mate with daughter cells took place 5% of the time, and only the final 1% of the time mating took place between different tetrads. (Tsai et al 2008). This is in stark contrast to *S. cerevisiae* where levels of outcrossing have been measured to as high as

20% (Goddard 2009). Hence, while higher levels of diversity are found in *S. paradoxus* due to the very low levels of crossing between lineages (Johnson et al 2004), there are higher levels of heterozygosity found in *S. cerevisiae* as a result of its higher level of outcrossing (Fay & Benavides 2005). Much of the research done in yeast has focused on *S. cerevisiae* so it was important to use this species in these experiments, to make sure that previous experiments and current literature could be used to draw valid conclusions from the results. However, by using *S. paradoxus* as well, it allowed us to get more insight into the actual natural mating behaviour of yeast without any implications of the long term domestication that *S. cerevisiae* has been subjected to.

Although clearing deleterious mutations through preferential mating is a theoretical good fit for yeast (Tazzyman et al in preparation) it has yet to be tested experimentally. I do this here by testing for preference for homozygous matings within the tetrad using spores in their ascus. I then try to identify the mechanism driving this preference by constructing artificial tetrads using vegetative haploid cells and by using tetrads of spores with their ascus removed.

5.3 Methods

5.3.1 Asexual fitness assay

The effect of the *ade1* mutation on viability was measured using an asexual fitness assay. The two competing strains (YCS 191 *MATa/α ho/ho lys2/lys2 ade1::KMX/ade1::KMX* Y55 and YCS 192 *MATa/α ho/ho*

lys2/lys2 ADE1/ADE1 Y55) were first streaked onto YEPD-agar (1% yeast extract, 2% bactopeptone, 2% glucose, 2.5% agar) and incubated at 30˚C overnight. A single colony from each strain was then used to inoculate separate tubes of liquid YEPD (1% yeast extract, 2% bactopeptone, 2% glucose). These tubes were then incubated for a further 24 hours with shaking in the assay conditions. 70μL of strain YCS 191 and 30 μL of strain YCS 192 were then used to inoculate a fresh tube of liquid YEPD. A sample of this mixture was then serially diluted and plated onto YEPD-agar. This gave an approximation of the initial numbers of each strain. The tube containing the mixture was then incubated with shaking for 24 hours. Then a sample was taken as before to give an approximation of the final numbers of each strain after asexual growth together. A measure of relative fitness of each strain was calculated by the ratio of their Malthusian parameters (Lenski et al 1991).

5.3.2 Strain preparation

Preferential mating within the tetrad was measured in *Saccharomyces cerevisiae* (YCS 173 *MATa/α ho/ho ade1::KMX/ADE1* Y55) and in *Saccharomyces paradoxus* (YCS 187 *MATa/α ho/ho ade1/ADE1* N17). To produce spores for the mate choice assays, both strains were plated on sporulation medium (2% potassium acetate, 0.87% complete mixture, 2.5% agar) and incubated at 25˚C for four days. Each diploid cell produced four spores encased in an ascus.

5.3.3 Mate choice assay

5.3.3.1 Spores in ascus

To test for preferential mating within the tetrad, sporulated cells were suspended in water, and streaked onto YEPD agar (1% yeast extract, 2% bactopeptone, 2% glucose, 2.5% agar). Whole tetrads were then isolated using a Zeiss micromanipulator microscope. These were then incubated at 30℃ for three hours. Plates were then checked every half hour for signs of completed mating, identified by the presence of one or two zygotes where a tetrad had been placed. The zygote cells were then separated and with the unmated cells were allowed to grow into individual colonies. Through mate switching, these become diploid, homozygous colonies. The status of the *ADE1* gene could then be identified as *ade1* homozygotes produce pink colonies This allowed easy identification of those cells which had mated in the cultured colonies.

5.3.3.2 Vegetative cells

To isolate the possible mechanism driving preferential mating, the mate choice assay was also carried out using vegetative haploid cells. Using vegetative cells eliminated any influence that the ascus or germination may have had on potential mate choice. This was carried out in *S. cerevisiae* and *S. paradoxus*. Here, tetrads were assembled on YEPD plates to mimic the structure of a tetrad without its ascus (Fig. 2.). For *S. cerevisiae* these artificial tetrads were arranged using a *MATa Ade1* (YCS190) cell, a *MATα Ade1* (YCS64) cell, a *MATa ade1* (YCS180) cell and a *MATα ade1* (YCS181) cell. For *S. paradoxus,* these tetrads were composed of a *MATa Ade1* (YCS188) cell, a *MATα Ade1* (YCS189) cell, a *MATa ade1*

(YCM158) cell and a *MATα ade1* (YCM159) cell (Fig. 2). Identifying which cells had mated with each other was done following the procedure used in the spore assay, due to the formation of pink colonies by *ade1* homozygotes.

5.3.3.3 Spores without ascus

To measure any effect the ascus may have on preferential mating within the tetrad, the mate choice assay was done exactly the same way as with the ascus. However, spores were suspended in a zymolyase solution for ten minutes. Tetrads free of their ascus were then isolated as before using a Zeiss micromanipulator microscope.

5.3.4 Identifying mating barriers

5.3.4.1 Effect of pheromone concentration on mating propensity

Mating propensity was measured as the proportion of tetrads in which mating was observed (either two or one zygotes being produced). This was done using *S. cerevisiae* ascospores as in the mate choice trials (YCS 173 *MATa/α ho/ho ade1::KMX/ADE1* Y55). Tetrads were plated on YEPD agar media as in the mate choice trials and incubated at 30℃ for three hours before being checked. In one treatment (spores), tetrads were treated as usual. In the second treatment (spores + exogenous pheromone), tetrads were plated onto YEPD agar that had had haploid *S. cerevisiae* cells growing on it for two days. This was to introduce exogenous mating pheromone in to the environment for the ascospores. Care was taken to place the ascospores in isolation from each other, as

well as the present haploid colonies. However, they were plated in a region where both **a** and **α** mating pheromone was present.

5.3.4.2 Effect of early zygote formation on intratetrad mating

Tetrads of the sporulated *S. cerevisiae* strain YCS173 were plated as with the mate choice assays. Twenty trials were followed in which early zygote formation was seen to bisect bisected the remaining two cells. Observation on these trials continued at 20-minute intervals until either mating or budding was observed from the previously unmated cells (Fig. 3.).

5.4 Analysis

5.4.1 Spore mate choice assays

The ratio of parental ditypes:non-parental ditypes: tetratypes was worked out using the formula:

$$cM = \frac{100}{2}\left[\frac{T + 6NPD}{PD + NPD + T}\right]$$ (Sherman 2002)

where cM represents the combined distance of *ADE1* and *MAT* genes from their respective centromeres, T represents tetratypes, PD represents parental ditypes, and NPD represents non-parental ditypes. When cM is smaller than 33.33, the ratio of parental ditypes:non-parental ditypes: tetratypes is skewed away from the 1:1:4 expected through independent assortment and recombination. Here, the combined distance was 34cM from their relative centromeres. So it was not small enough to

skew the expected ratios of parental ditypes:non-parental ditypes: tetratypes away from 1:1:4. This was important as it was only in the tetratypes where homozygous matings (with respect to the *ADE1* and *ade1* alleles) could take place in the mate choice assay (Fig. 1.).

When using spores for the mate choice assay, there were six possible combinations of spores within the tetrad (Fig. 1.). In two of these combinations, mating could only take place between a spore carrying the wild-type *ADE1* allele and one carrying the *ade1* mutant allele. For the other four combinations, each spore had the potential to mate with either a spore carrying the wild-type *ADE1* allele, or an *ade1* mutant. The calculated expected number of homozygous (both *ADE1* and/or both *ade1* spores form mating pairs) and heterozygous (only matings between *ADE1* and *ade1* spores) matings took into account the two combinations that only allowed for heterozygous matings. If mating was random with respect to the *ADE1* gene, we expected 2/3 of all matings to be between a wild type and a mutant spore (1/3 coming from the 2 combinations where spores don't have a choice, the second 1/3 coming from half of the 4 combinations with potential choice resulting in wildtype to mutant matings). This left an expected 1/3 of matings to have resulted in homozygous zygotes. The observed numbers of heterozygotes and homozygotes were tested against the null hypothesis that mating was random with respect to the allele at the *ADE1* locus using a contingency table.

Measures of preference for homozygous matings were calculated as the proportion of homozygous matings from the total number of

matings where choice was present. In 1/3 of the trials no choice was present so matings resulting in homozygotes could not have taken place. Therefore, preference = total number of homozygous matings/[(2/3)*total number of observed matings]. This should have a value between between 0 to 1. A value of 0 indicates that only matings resulting in heterozygotes occurred, i.e. there was complete preference for heterozygous mating. A value of 1 indicates mating resulting in homozygotes always occurred when they were possible, a complete preference for homozygous mating. And a value of 0.5 indicates that mating was random with respect to the allele at the *ADE1* locus..

5.4.2 Vegetative cells mate choice assays

Because I used vegetative haploid cells of known mating type and genotype, I could assemble tetrads in which all cells had the choice of homozygous or heterozygous matings, in contrast to the natural tetrads (see Spore mate choice assays, above), 1/3rd of which do not allow any choice.

If mating was taking place at random with respect to the *ADE1* gene, homozygous mating will have taken place in ½ of all trials. The observed numbers of heterozygotes and homozygotes were tested against the null hypothesis that mating was random with respect to the allele at the *ADE1* locus using a contingency table

Measures of preference for homozygous matings were calculated as the proportion of total trials where homozygous mating was observed. As every trial had the potential for homozygous mating, this is simply

stated as = total number of homozygous matings/total number of observed matings. As above, this gave a number between 0-1. A value of 0 indicates complete preference for heterozygous mating, a value of 1 indicates a complete preference for homozygous mating, and a value of 0.5 indicates non-preferential mating.

5.5 Results

5.5.1 Asexual fitness assay

The asexual fitness assay showed that the *ADE1* gene causes a drastic effect on asexual fitness when not expressed. This assay measured the *ade1* mutant strain to have a relative fitness of 0.475 (n=5, standard error = 0.043) to the *ADE1* strain.

5.5.2 Spores in the ascus

The mate choice assays using spores still encased in their ascus, showed that non-random mating was taking place with respect to the *ADE1* allele. Homozygous matings were observed significantly more often than expected if mating were taking place at random. This was true for both species. *Saccharomyces cerevisiae* resulted in homozygous matings 173 times from the 413 trials in which mating was observed. This is significantly more than the expected value of 137.7 (*cerevisiae* χ^2=9.335, DF=1, p=0.002) and represents a preference for homozygous matings of 0.628. *Saccharomyces paradoxus* resulted in homozygous matings 40 times from 80 trials in which mating was observed. This is significantly

more than the expected 26.67 one would find if mating were non-preferential (χ^2=10, DF=1, p=0.002) and represents a preference for homozygous matings of 0.75.

5.5.3 Vegetative cells

When the mate choice assay was performed using artificially constructed tetrads from vegetative cells, both species showed similar levels of preference for homozygous matings. In *S. cerevisiae* 34 trials resulted in homozygous matings from a total of 52. This is significantly higher than the expected 26 of the null hypothesis of random mating (χ^2=4.923, DF=1, p=0.027) and shows a preference of 0.654 for homozygous matings. *S. paradoxus* resulted in homozygous mating 28 times from the 40 trials in which mating was observed. This is significantly higher than the expected 20 (χ^2=6.4, DF=1, p=0.011) and shows a preference for homozygous matings of 0.7.

5.5.4 Spores without the ascus

When the ascus was removed, non-random mating was still observed in both species. *S. cerevisiae* displayed homozygous mating 187 times from the 456 trials in which mating was observed. Significantly higher than the expected 152 (χ^2=9.938, DF=1, p=0.002), showing a preference for homozygous matings of 0.615. *S. paradoxus* displayed homozygous mating 42 times from the 80 trials in which mating was observed. Significantly higher than the expected 26.67 (χ^2=13.225, DF=1, p<0.001), showing a preference for homozygous matings of 0.788.

5.5.5 Mating propensity

When mating was observed, the prevalence of complete mating was seen to be higher in *Saccharomyces cerevisiae* both with the ascus (*S. cerevisiae* = 60% *S. paradoxus* =2% χ^2=1716.33, DF=1, p<0.0001)and without the ascus (*S. cerevisiae* = 44% *S. paradoxus* =25% χ^2=19.25, DF=1, p<0.0001). In cases of incomplete mating, only one mating pair was observed with the remaining two spores budding daughter cells (Table 2).

5.5.6 Comparison of preference for homozygous matings

Overall there was a significant preference for homozygous matings across both species (preference = 0.688 χ^2=14.440, DF=1, p<0.001). Both species displayed significant levels of preference for homozygous matings (*S. cerevisiae* preference = 0.651 χ^2=9.000, DF=1, p=0.003, *S. paradoxus* preference = 0.868 χ^2=54.760, DF=1, p<0.001). However, *S. paradoxus* had a significantly stronger preference to produce homozygotes than *S. cerevisiae* (χ^2=21.275, DF=1, p<0.001). The stronger preference in *S. paradoxus* was present both in spores in the ascus (χ^2=7.68, DF=1, p=0.006) and in spores without the ascus (without ascus χ^2=17.42, DF=1, p<0.001) but not in vegetative cells (χ^2=1.19, DF=1, p=0.275). In *S. cerevisiae* there was no significant difference between the level of preference seen in spores and the strength of preference measured in vegetative cells (χ^2=0.396, DF=1, p=0.529). In *S. paradoxus*, while there was no significant difference between the level of preference measured in

spores and that measured using vegetative cells (χ^2=2.333, DF=1, p=0.127), there was in fact a significant difference in strength of preference between spores without the ascus and vegetative cells (χ^2=4.882, DF=1, p=0.027)

5.5.7 Effect of pheromone concentration on mating propensity

When no exogenous pheromone was present, only 10% of plated tetrads produced matings. When pheromone level was higher due to exogenous pheromone there was a significant increase in matings with 36.5% of plated tetrads having matings (χ^2=29.34, DF=1, p=0<0.001). (Fig. 5.).

5.5.8 Effect of early zygote formation on intratetrad mating

In all of the trials followed, the two unmated cells proceeded to bud daughter cells rather than mate with each other. When the early formation of a zygote bisects the other two cells in a tetrad, it creates an impassable barrier preventing any further mating.

5.6 Discussion

5.6.1 Mate preference

My results support the theory that sexual signalling and mate choice help to clear deleterious mutations from Saccharomyces populations. I found that homozygous offspring were significantly in

excess in both species, whether in natural tetrad ascospores, or in artificial tetrads composed of four spores or four vegetative cells (Fig. 4.). By implication, this suggests that mate choice favours assortative coupling of spores by quality.

Saccharomyces paradoxus displayed a higher preference for homozygous matings compared to *Saccharomyces cerevisiae*. A possible explanation for this is that the two species differ in germination time. For *S. cerevisiae* and *S. paradoxus*, the time taken for germination to be completed is likely to be very species specific as it forms a prezygotic barrier to reduce the frequency of costly interspecies matings (Maclean & Greig 2008). The time taken for a spore from *S. cerevisiae* to form a bud (the early stages of mitotic growth) is 4.79 ± 13.18 hours (n=10). The same process takes 9.1 ± 27.57 hours (n=10) in *S. paradoxus*. These timings include the time taken for germination as well as the early stages of mitotic growth, so can only be used as estimates for relative germination times. As germination and the early stages of the life cycle take longer in *S. paradoxus* ($t=26.75$, $DF=18$, $p<0.0001$), it may help exaggerate any signalling effects by allowing more time for the mating pheromone to be produced and secreted as well as giving it a longer window of opportunity to act on nearby cells. As the process seems to take place faster in *S. cerevisiae*, the difference in perceived signal strength between a wild type and mutant haploid may not be as large as in *S. paradoxus*. This view is supported by the observation that the weakest difference in preference for homozygous matings was seen in trials using vegetative cells (Fig. 4.), principally due to a decline in *S.*

paradoxus mating preference under this condition. Vegetative cells do not germinate, so this lengthy time period is absent in this trial, but present under the other two conditions where spores were used.

5.6.2 Inter-tetrad mating & purging of deleterious mutations

Mathematical modelling shows that preference for homozygous matings at rare intervals of sexual reproduction is a theoretically plausible strategy for *Saccharomyces* to adopt (Tazzyman et al in preparation). The results from these experiments support this theory, as both *S. cerevisiae* and *S. paradoxus* display clear preference for homozygous matings. However, without a fuller understanding of yeast ecology it is difficult to design either a model or experiment to accurately represent the choices available under realistic mating conditions.

Early work in *Saccharomyces* yeast suggesting that mating took place within the ascus itself [Miller & Hoffmann-Ostenhof 1964], and more recent work into population genetics, has given rise to the strong belief that it is a highly inbred organism [Tsai et al 2008]. Intuition suggests that the inbreeding is due to high levels of intra-tetrad matings. However, intra-tetrad mating may not actually take place as often as previously assumed. In 2010 Murphy & Zeyl showed through mating assays (as opposed to using genomic data to make estimates) that contrary to traditionally held beliefs, there is actually an unexpectedly high level of inter-tetrad matings that take place in both *S. cerevisiae* and *S. paradoxus*. They argue that neighbouring tetrads are likely to be

descended from clonal siblings anyway, so inter-tetrad mating will still result in high levels of observed inbreeding at a population genetic level.

This inter-tetrad mating offers another option that could be included in Tazzyman's (in preparation) multi locus model. By having the ability to mate with a spore from a neighbouring tetrad, the probability of finding a high quality mate increases, as now there are more than just two spores to choose from. Similarly, the ability to mate outside of one's own tetrad may have an effect on the results presented here. In these mate choice assays, each trial consisted of one tetrad in isolation. As shown in Fig. 1., two of the six possible spore combinations do not allow for homozygous matings. If in these instances, the option to mate outside of the tetrad was available, they may also have opted for homozygous matings. In calculating preference for homozygous matings, the two combinations that did not allow for mate choice with respect to the *ADE1* gene were taken into account and compensated for. So while allowing for inter-tetrad mating may affect the results, it is unlikely that it will impact them greatly. It is however important to include this option in any future model looking at the purging of deleterious mutations in *Saccharomyces*.

5.6.3 Complete mating in the tetrad

One of the big problems with modelling the behaviour of *Saccharomyces* is the distinct lack of knowledge surrounding yeast ecology and mating behaviour in general. In Tazzyman's (in preparation) model there are three mating scenarios. Diploids can either wait and reproduce asexually, or undergo meiosis. Having produced spores, these

can then either mate randomly or with choice (with respect to homozygous matings). This again does not represent the full range of options open to yeast.

While the main purpose of the experiments presented here was to determine whether there was preference for homozygous mating within the tetrad, they did also help shed some light on *Saccharomyces* mating behaviour. Table 2 shows that complete mating (all four spores mate to produce two diploid zygotes) does not always take place. Indeed there was no guarantee that any mating would necessarily take place at all before haploid cell division. When mating was observed, the prevalence of complete mating, where all four cells in the tretrad mated, was seen to be higher in *Saccharomyces cerevisiae*.

The difference in observed instances of complete mating can be attributed to various different factors. It may simply be that *Saccharomyces cerevisiae* are more willing to mate. This would make sense in terms of their evolutionary histories, because *S. cerevisae* is a domesticated species, and the strain used here (Y55) is a long established laboratory strain. It is possible that in this artificial environment, the laboratory strain of *S. cerevisiae* may have been artificially selected for fast matings, in the process losing some of its ability to discriminate mate quality, an ability that is retained in the wilder strain of S. paradoxus.

There are several factors that are equally applicable to both species that will lead to some spores budding daughter cells rather than mating with a partner. Firstly, there needs to be a sufficient base level of pheromone for mating to take place. Mating propensity can increase

significantly in spores when tetrads are flooded with exogenous pheromone (Fig. 5.). In the mate choice assays, testing for preference for homozygous matings, carried out here tetrads were placed in isolation. If they were to be placed closer together, each cell would be subject to more pheromone than when tetrads are isolated. The mating pheromone causes cell cycle arrest. It is in this period that cells make their choice in mating partners. So if not enough pheromone is present to keep them in this state, they will just proceed into mitosis.

The experiments here were all carried out at optimal growth conditions. This is not necessarily a fair or accurate representation of the mating environment encountered by wild *Saccharomyces*. For mating to take place, there not only needs to be enough pheromone being produced, but it also needs enough time to diffuse towards other cells. In an environment that supports slower growth, there will be a longer period of time for the pheromone to act. Not only will it have longer to diffuse, but due to the slower growth rate, cells will also take longer to break it down. So while these experiments show a seeming resistance to mate, it may just be a by-product of an optimal growth rate of artificial laboratory conditions.

5.6.4 Single matings in the tetrad

In all mate choice assays there were several tetrads that displayed a single mating. In these cases two spores would fuse to form a diploid homozygote, leaving the remaining two spores to bud daughter cells. Three possible explanations can be attributed to this occurrence. Firstly,

as mentioned above, willingness to mate may play an important factor here. If cells are not fully prepared to mate due to a lack of mating pheromone, then they will simply not do so. This suggests that in some instances some cells are subject to a high enough level of mating pheromone, while others are not, leading to only a single mating within the tetrad.

Cells are attracted to the strongest source of mating pheromone. In the scenarios where there is mate choice with respect to the *ADE1* allele (Fig. 1.), two cells will be attracted to the same potential mate. For example a wild type and a mutant *MATa* cells will both be attracted to a wild type *MAT*α cell. Here, the *MAT*$\boldsymbol{\alpha}$ cell has a choice of two potential partners. When it commits to fusing with one of the two *MATa* cells, the rejected cell will still be in cell cycle arrest attracted to the *MAT*α cell until it has broken down enough of its mating pheromone. Once it has stopped being attracted to the now mated cell, it can only mate with an unattractive *MAT*α cell. However, this mating can only take place if both remaining cells of the tetrad are still in sync. If either one has exited cell cycle arrest or subsequently gone beyond the G1 phase, then mating can no longer take place. This would result in one diploid zygote, and two haploid cells budding daughter cells.

The third process leading to a single mating is a physical barrier. The tetrad consists of four tightly packed spores. After germination takes place, all four cells are still in contact with each other (Fig. 1.). This is useful because, as all cells are equidistant from each other, differences in

signal strength should be due to amount of pheromone being produced rather than distance from the source. However, this tight arrangement can also impede mating. In cases where a zygote is formed that bisects the remaining two cells (Fig. 3.), no further mating can take place within that tetrad.

5.6.5 Incomplete mating in the wild

The prevalence of unmated cells is reasonably high in these experiments (Table 2). This cannot necessarily be regarded as something that takes place as part of the natural mating behaviour of yeast. The experimental set up deals with tetrads in isolation. In the wild it is reasonable to presume that ascospores are not always isolated, and are likely to be found in groups. This has some important implications for the natural mating behaviour of yeast. We known that enough mating pheromone needs to be present for mating to take place (Fig. 5.). There may not be enough being produced by a single tetrad. With several tetrads surrounding each other, each cell will be subject to more mating pheromone. So more matings will take place.

In the instances where cells are left without viable mates within their own tetrad, they are seen to bud daughter cells in these experiments. Presumably, they will then go on to switch mating type and mate with each other after a further generation. This act of selfing would provide a very easy and obvious explanation for the high levels of

inbreeding that are expected to take place in yeast. This is particularly true of *Saccharomyces paradoxus. S. paradoxus* has very low levels of heterozygosity, so is expected to have high levels of inbreeding. The results show that tetrads with observed matings usually only had one zygote and two budding cells. With so many cells being left to self, it would certainly suggest a high level of inbreeding. But, in the wild, tetrads are unlikely to be quite so isolated. Instead, when a cell is left without a mate from its own tetrad, it will still have the choice to mate with a cell from a neighbouring tetrad in a similar situation.

This now leaves us with a tricky situation to explain. If *Saccharomyces cerevisiae* cells mate within their tetrad more than *Saccharomyces paradoxus*, then why does *Saccharomyces paradoxus* display higher signs of inbreeding? Here the main result from these experiments can offer a solution. While it is true that *S. cerevisiae* seemed to show more cases of complete mating, the strongest preference for homozygous matings was observed in *S. paradoxus*. So while *S. cerevisiae* tend to mate within their own tetrad more often, there are still a large proportion of these matings that result in heterozygosity. *S. paradoxus* on the other hand are more discerning when it comes to choosing mates. The experiments show that *S. paradoxus* has a higher tendency to produce homozygous matings within the tetrad. This still leaves us with a lot of unmated cells, with only potential partners from neighbouring tetrads. How does this lead to low levels of heterozygosity? Neighbouring tetrads are likely to be very similar. Indeed the founding diploid cells from which

these tetrads descend are almost certainly going to be clones of each other. So an unmated cell from one tetrad is likely to find a mate from a neighbouring tetrad that is very genetically similar to it. In the case where more than one potential partner is available outside of the tetrad, we have already seen that *S. paradoxus* has a preference for homozygous matings. So this should manifest itself as similar cells mating with each other leading to the high levels of perceived inbreeding found in *S. paradoxus*.

5.6.6 Conclusion

It has previously been shown through mathematical models that preference for homozygous matings within the tetrad is a theoretically plausible strategy for *Saccharomyces* to adopt. The results presented here show that not only is this theoretically plausible, but that it can and does take place in practice.

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5.8 Figures

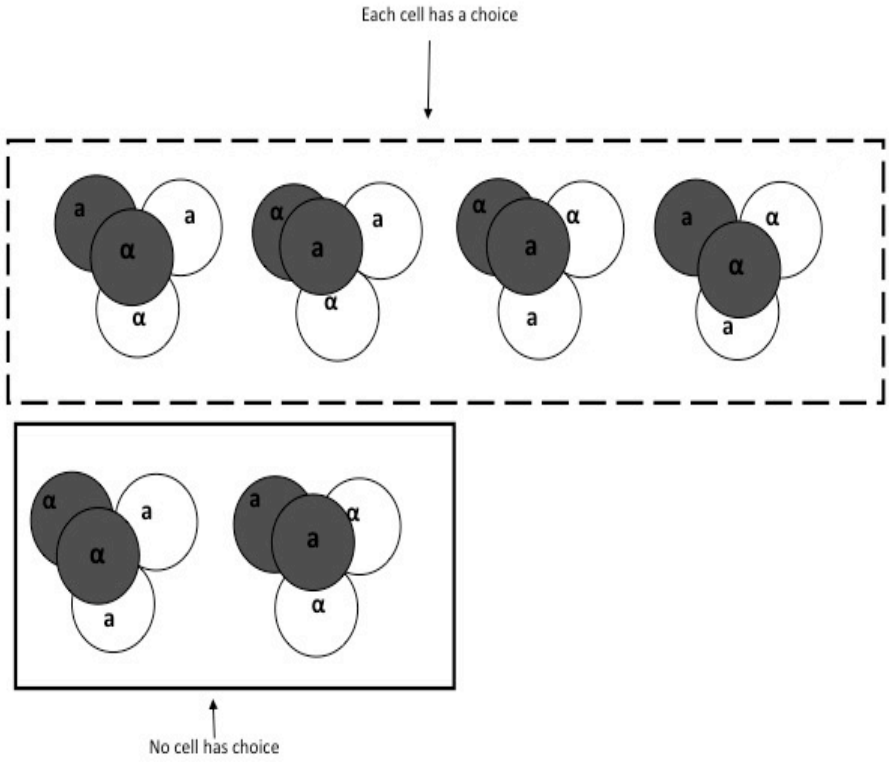

Figure 1.

Six possible permutations of spores within the tetrad.

Dark circles represent spores carrying the *ade1* mutation. White circles represent spores with the wildtype ADE1 allele. *MATa* spores are marked by the letter **a**. *MATα* spores are marked by an **α.** Choice is only present in four of these combinations. In the remaining two combinations, only heterozygous zygotes can be produced. If mating is taking place at random, we expect wild type to heterozygous matings where the cell has no choice, and in half of the trials where the cell has a choice. Thus in total we expect 2/3 of selections to favour heterozygous matings if no preference is present. Deviation from this expected value determined whether preferential mating took place using a χ^2 test.

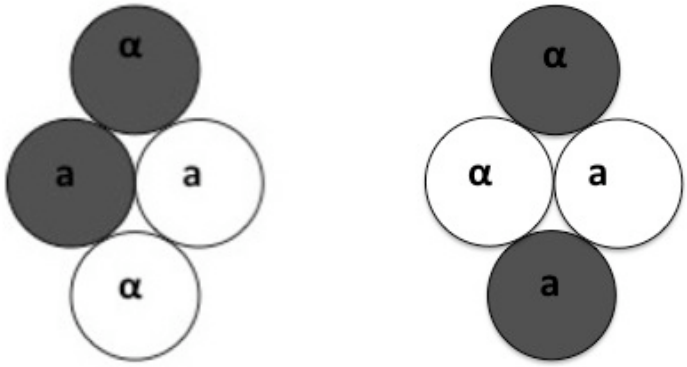

Figure 2.

Artificial tetrads made using vegetative haploid cells. Dark circles represent spores carrying the *ade1* mutation. White circles represent the wild type spores. *MATa* spores are marked by the letter **a**. *MATα* spores are marked by an **α.** As each cell always has a choice in mating partner, we would expect homozygous mating ½ of the time. Deviation from this expected proportion of matings was tested using a χ^2 test.

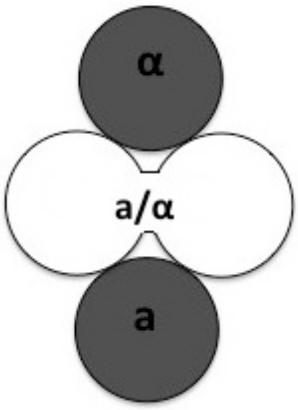

Figure 3.

Early zygote formation bisecting remaining two spores. Dark circles represent spores carrying the *ade1* mutation. An "*α*" denotes a *MATα* spore. An "a" denotes a *MATa* spore. The joined white circles represent a zygote of mating type *α*/*a.*

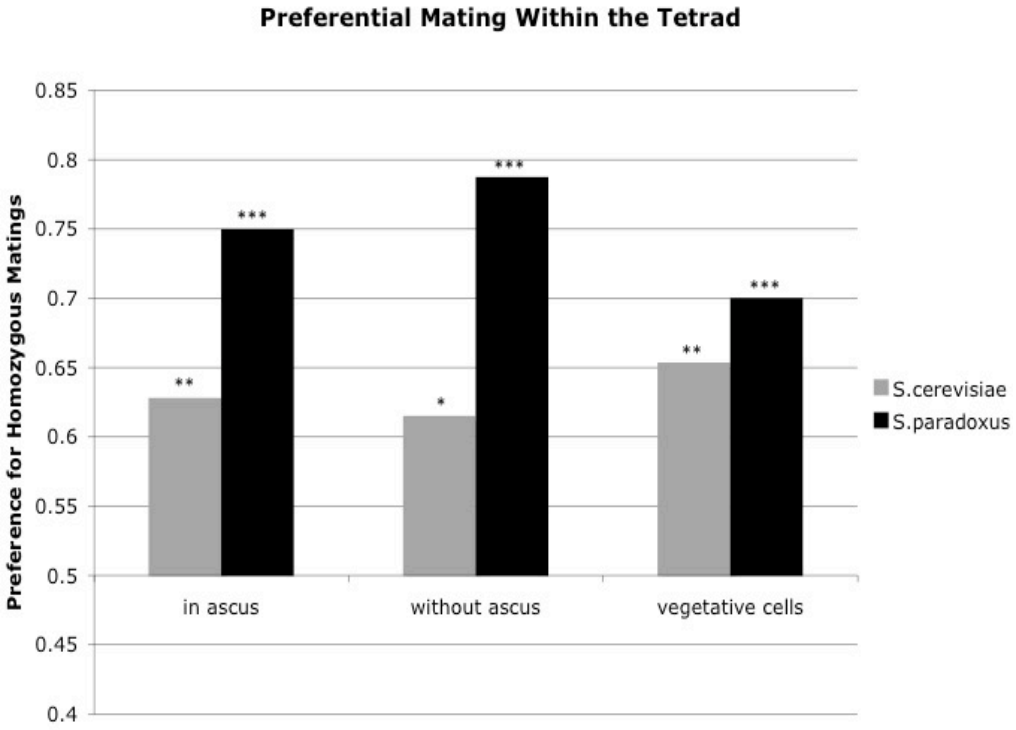

Figure 4.

Preference for homozygous mating. The graph shows a clear preference for homozygous matings in both species under all conditions assayed. The measure of preference was calculated as the proportion of homozygous matings from the total number of mated trials in which this was possible. For trials using spores, preference = total number of homozygous matings/[(2/3)*total number of observed matings]. As homozygous matings were always possible when using vegetative cells, for those trials preference = total number of homozygous matings/ total number of matings. This gave a number between 0-1. A value of 0 indicates preference for heterozygous mating, a value of 1 indicates a complete preference for homozygous mating, and a value of 0.5 indicates

non-preferential mating. Stars represent the level of probability that the observed preference for homozygous mating did not occur by chance (*= $p<0.01$, **=$p<0.001$, ***=$p<0.0001$)

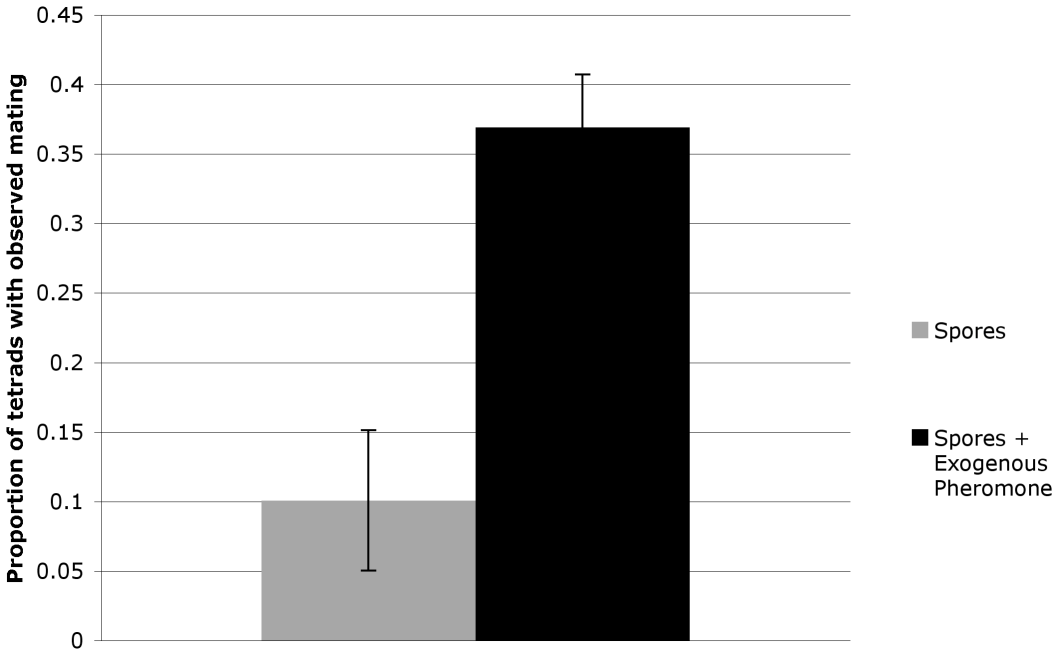

Figure 5.

Mating propensity increases with concentration of mating pheromone. Tetrads of spores were plated on YEPD-agar and allowed to mate. In the 'spore' treatment, the conditions were the same as with the mate choice trials. In the 'spore + exogenous pheromone' treatment the YEPD-agar also contained exogenous *a*-pheromone and *α*-pheromone. Tetrads were then observed for matings (either one or two). The results show that the presence of extra pheromone in the mating environment increases mating propensity. The error bars represent the standard error for the data set.

5.9 Tables

	Total Number of tetrads with matings	Hetero-zygous matings	Homo-zygous matings	Expected hetero-zygous matings	Expected homo-zygous matings	χ^2	p	DF	Prefe-rence for homo-zygous mating
Spores									
S.cerevisiae in ascus	413	240	173	275.3	137.7	9.335	0.0022	1	0.6283
S.cerevisiae without ascus	456	269	187	304	152	9.938	0.0016	1	0.6151
S.paradoxus in ascus	80	40	40	53.33	26.67	10	0.0015	1	0.75
S.paradoxus without ascus	80	38	42	53.33	26.67	13.225	0.0003	1	0.7875
Vegetative Cells									
S.cerevisiae	52	18	34	26	26	4.923	0.0265	1	0.6538
S.paradoxus	40	12	28	20	20	6.4	0.0114	1	0.7

Table 1.

Results of mating trials. For trials using spores, the expected frequency of homozygous matings was 1/3 (see methods and Fig. 1.). Preference for homozygous matings was calculated as: preference = total number of homozygous matings/[(2/3)*total number of observed matings]. This took into account possible mating in tetrads where homozygous mating was not an option (see methods and Fig. 1.).

For trials using vegetative cells, the expected frequency of homozygous matings was ½ (see methods and Fig. 2.). Preference for homozygous matings was calculated as: preference = total number of homozygous matings/total number of observed matings. Each tetrad had the potential for homozygous mating, so the measure of preference is unadjusted (see methods and Fig. 2.)

	Total number of tetrads with matings	Total number of tetrads with complete mating	Total number of tetrads with a single mating
Spores			
S. cerevisiae in ascus	413	241	172
S. cerevisiae without ascus	456	154	302
S. paradoxus in ascus	80	2	78
S. paradoxus without ascus	80	20	60
Vegetative Cells			
S. cerevisiae	52	17	35
S. paradoxus	40	6	34

Table 2

Not all cells mate. From all the tetrads set up, a large proportion were observed to produce only a single zygote. In these cases, only two cells had fused together to form a diploid, while the remaining two cells remained independent of each other to bud mitotic daughter cells.

6. General Discussion

6.1 Overview

Saccharomyces cerevisiae offers unique possibilities as a model system for evolutionary research. It has the practical advantage of being very easy to keep and maintain in the limited space available under laboratory conditions. Aside from being cost effective, it is also very practical for experimentation. It can be manipulated with great ease by changing environmental conditions, or by manipulating its genome. But perhaps the biggest advantage of using *S. cerevisiae* as a model system in evolutionary biology is that it is a eukaryote with relatively short sexual and asexual generation times. This not only allows us to witness evolution in real time, but also allows us to have multiple replicates with identical starting populations. However, there is still some important work that needs to be done to keep developing yeast as a research tool. Although *S. cerevisiae* is becoming a more popular model organism in evolutionary biology because of these benefits, its ecology is still poorly understood. Without knowing how or why yeast behaves in nature, it is impossible to put experimental results into an evolutionary context. In this thesis I have attempted to further our understanding in this area by characterising the cost of sexual signalling in *S. cerevisiae* and then investigate why such a costly mechanism might have evolved.

Competition over mates can have a significant impact on morphology, behaviour and life history. The evolution of exaggerated traits due to sexual selection is especially intriguing as it often goes against natural selection (Searcy 1979). While sexual selection in itself can be a costly process, it is also a consequence of sexual reproduction.

While there are theoretical benefits to sexual reproduction (e.g. enhanced adaptation, the mutational deterministic process, DNA repair), it is still a very costly form of reproduction. If two competing populations were identical in every respect except how they reproduced, the asexual population would out compete the sexual population very quickly. This supposes that asexual and sexual reproduction both take similar amounts of time, and individuals from both populations produce the same number of offspring. Sex is also interesting in that it seems to act against natural selection. Specific combinations of alleles that have been shown to be beneficial by making it through several rounds of selection are then split up to make different combinations as a result of recombination.

While sex is so costly at a population level, there can also be remarkable costs at an individual level. Competition for and the selection of mates have lead to some remarkable characteristics in some organisms. In simple terms Darwin's theory of selection suggests that survival favours those individuals that are best adapted to their environment. But it was Darwin himself who noted that his own theory could not explain the appearance of exaggerated secondary sexual characteristics such as the peacocks elaborate tail feathers. After his initial comment on the subject in his book 'On the origin of species' (1859), where he proposed that sexual selection could account for such extravagant traits, he later published a book dealing entirely on the subject of sexual selection in 1871. The study of sexual selection has remained an enticing topic ever since. In more modern times, with our detailed understanding of genetics and advanced mathematical modelling techniques, several theories have

been put forward to answer some of the persisting questions about sex. Why did sex evolve if it is so costly and how did it lead to exaggerated sexual displays? A century and a half on from Darwin's first published mention of sexual selection, and we still don't have many definitive answers. The yeast *Saccharomyces cerevisiae*, with its unique attributes, offers a chance to make further substantial progress towards finding these answers.

The experiments presented in this thesis are a step in this exciting new direction. They also show how simple experimental designs can generate clear results providing specific answers to specific questions. I will now provide my main findings and suggest possible future experiments to keep progressing our understanding of sex and sexual selection using *Saccharomyces cerevisiae* as a model system.

6.2 Evolution of sexual signalling

6.2.1 The cost of sexual signalling in yeast

I have shown that the mating pheromone of *S. cerevisiae* is costly to produce. This cost is affected by the phenotypic and genetic quality of the signaller, so that the cost of producing the pheromone is much smaller for high-quality signallers than for low-quality signallers. Low-quality individuals were shown to produce weaker signals than high-quality individuals under identical experimental conditions. These results are consistent with Zahavi's handicap theory (1975, 1977) on the evolution of sexual displays.

Although the cost is now quantified, the details of what this actually represents remains unknown. The cost could be due to a number of factors. We know that reducing the amount of pheromone produced by knocking out MFa/α genes increases fitness (Smith & Greig 2010). But it would also be interesting to know the effects of over expression of the pheromone. The results of this may be quite telling. Will over expression lead to a fitness decrease as expected? More importantly, will the signal strength actually increase? If the pheromone signal evolved as a conditional handicap, then any increase in the signal strength should come at a very significant cost such as decreased viability. If the signal strength does not increase, some interesting research could be carried out. By testing for the precursors of the mature mating pheromone, you could begin to see where some of the cost of signalling actually takes place. By comparing results with a cell signalling at normal strength, you could see if the precursors are present in larger quantities when the pheromone encoding genes are over expressed. The cost of signalling is likely to come from either the production of the pheromone itself, in the secretion of it or a combination of the two. Smith & Greig (2010), show that there is a difference in cost of the two mating pheromones. While this may be down to experimental error, it may be indicative of the different way in which they two pheromones are produced and secreted (Kurjan 1992).

It may also be possible to assess how much of the cost is due to secretion of α-pheromone by measuring the cost of other proteins that use the same classical secretory pathway such as Yck2 (Babu et al 2002).

A similar cost for similar amounts of protein may indicate that the majority of the cost is due to the secretion of the α-pheromone. If the cost of associated with Yck2 is significantly smaller than that of α-pheromone, then the difference will be in the production of the pheromone itself.

A longer way, but perhaps more powerful method of analysing the cost of signalling in *Saccharomyces cerevisiae*, would be to isolate genes crucial in the production or transport of the pheromone. Knocking them out individually, and comparing the asexual fitness as described by Lenski (1991) of such a strain to a wild type strain would begin to show where the cost is being incurred. For example, *STE6* could be knocked out in a *MATa* strain. This gene is essential for the production of **a**-pheromone and encodes a protein that transports the pheromone across the plasma membrane (Kuchler et al 1989). By knocking out genes such as these and measuring their effects on fitness, the cost of the processes involved in sexual signalling in yeast can begin to be seen in greater detail.

In the experiments carried out by Smith & Greig (2010), the fitness of strains was altered by introducing auxotrophic markers. A reduction in fitness lead to a reduction in signal strength. It is remarkable that simply knocking out nutrient genes can have such a significant effect on fitness and in turn pheromone signalling. It would be interesting to assess these markers one by one for their effects on fitness and signalling to further understand the pleiotropic effects that cause fitness to be linked so heavily with sexual signalling. These auxutrophic markers are not necessarily what one might find in wild populations. Homozygosity in wild populations seems to be quite common (Tsai et al 2008). Testing for

covariance of fitness and signal strength in wild populations is therefore a crucial next step, to identify how mate choice might work in nature.

Environmental effects are also worth investigating. Harsh conditions can have an impact on the cost of signalling (Smith & Greig 2010). Most laboratory work is carried out on a complete medium and incubation at optimum growth conditions. This is unlikely to be representative of wild conditions. As shown by the variation in fitness due to auxotrophic markers, the presence or absence of nutrients can have a big effect on viability and in turn signalling. This is another area of research that could help yield some very interesting results in itself as well as helping the design of more realistic experimental studies.

The cost of sexual signalling in yeast is characteristic of a sexual signal evolved as described by the handicap principle (Zahavi 1975, 1977). While the result lends support to this theory, it should by no means be at the expense of Fisher's theory of runaway selection (1930). The cost of signalling outlined here is consistent with one of Fisher's requirements. The trait has to be costly for it to stabilise. So the over abundance of mating pheromone produced by *S. cerevisiae* could still have evolved as outlined by Fisher. Testing Fisher experimentally from start to finish would be difficult but, given enough time and patience, it may be possible. Choosing a variable and heritable trait would probably be the simplest step. Such a trait may be another secretory protein such as the enzyme invertase that hydrolyses sucrose (Meyer & Matile 1975, Carlson & Botstein 1983). But building up a heritable preference for such a trait would be a very lengthy process that may or may not even work. It has

already been shown in *S. cerevisiae* that a strong signaller can invade a population of weak signallers (Rogers & Greig 2009); so showing how an attractive trait and the preference for it evolve into selection for an exaggerated sexual trait should be the next step. Given the benefits of evolutionary experimentation in yeast and the ease with which they can be manipulated at a genetic level, *S. cerevisiae* probably represents the best chance at demonstrating Fisher's process experimentally.

6.3 Evolution of sexual selection

6.3.1 Preference for mate size in *Saccharomyces cerevisiae*

My experiments showed that mate size represented a direct benefit that could be selected for in yeast. In rich environments, large cells had a higher initial fitness, where on a poor environment small cells had a higher initial fitness. Such a potential benefit should result in the evolution of mate choice for size. The results of the mate choice assays confirmed this to be the case. Large mates are preferred on rich medium while small mates are preferred on poor medium. However, a more detailed breakdown of the results showed some interesting behaviour. Large spores had a similar level of preference for the best available mate in both environments. Comparatively, small spores on the other hand displayed a much higher preference for large mates in the rich environment than they did for small mates in the poor environment. In both environments, it was the least desirable sized cells that displayed the strongest preference; small cells showing the strongest preference in the rich environment, and large cells showing the strongest preference in the

poor environment. These results are telling, in that they suggest that there is more to mate choice than simply cells signalling their attractiveness through different concentrations of mating pheromone.

Initial rate of mitotic growth was measured for both spore types in both environments. This was done to get an estimate of germination time. Large spores grew faster than small spores on the rich environment and small spores grew faster than large spores on the poor environment. This measurement may explain the differences in observed preference in the mate choice trials. A faster germination time represents a spore being ready to mate first. In trials where the undesirable spore size was given the choice of mate, the single desirable spore germinated first. For example, in the rich media, when small spores were given the choice of mate, the large spore germinated first. By the time one of the small spores germinated, the large spore will have produced enough pheromone to make it a very attractive mate. So the small spore will mate with the large spore. Even if both small spores finish germinating at the same time, the large spore will be the most attractive mate as it had been producing mating pheromone for a longer period of time. The reverse situation will be true in the poor environment where small spores germinated faster.

Readiness to mate may also account for the lower preference seen when the desirable mate was given the choice of mating partner. On the rich environment, two large spores germinated faster than the small spore. So the majority of the time, the two large spores will mate. However, mating will not always take place. As both spores will have just emerged from germination, neither will be particularly attractive, as they

will not have been producing pheromone for very long. Should one of the two large cells go on to bud a daughter cell, the remaining large cell now becomes an attractive mate for the freshly germinated small spore. This accounts for the observed matings between small and large spores in the trials where large spores were given choice.

In the poor environment, small spores only had a negligible preference for small mates. A combination of germination times and overall slower growth rate in the poor environment can explain this. When in a phenotypically poor state, the cost of sexual signalling increases, as is the case of cells in the poor environment. When two small spores germinate, they will not be very attractive, and will take far longer to produce pheromone to the same concentration as those in the rich environment. Thus, two small mates in a poor environment are less attractive to each other than two large mates in a good environment. This leads to fewer small-to-small matings when small spores are given the choice of mate in the poor environment.

6.3.2 Preferential mating observed in intratetrad matings of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*

There are already mathematical models that support the idea that *Saccharomyces* yeasts use their ability to reproduce sexually to clear out deleterious mutations, which may have built up in the genome during several cycles of mitotic growth (Tazzyman in preparation). For this to work, good quality mates would have to mate with each other where possible, rather than mating indiscriminately. These experiments were

designed to test whether such preferential mating could take place in practice. Two diploid strains were created, one in *Saccharomyces cerevisiae* and one in *Saccharomyces paradoxus*, which produce two good quality spores and two poor quality spores through meiosis. Preferential mating was tested using tetrads of spores still cased within their ascus. This was in an effort to give each cell an equal opportunity of mating with any potential mate, and to replicate a plausible scenario that cells may find themselves in when they mate in the wild. Mate choice assays were also set up by artificially building tetrads using either spores or vegetative haploid cells. In all cases there was significant preference for the two good quality cells to mate with each other. This was observed in both species. The shown preferential mating supports the theory that yeast undergoes sex to clear deleterious mutations from the population.

As the mating pheromone is an honest signal, good quality mates will produce more pheromone than poor quality mates. The two strongest signallers mate together, which will also be the two best quality mates. It is interesting to note that there was a stronger preference for mate quality measured in *S. paradoxus* than in *S. cerevisiae*. When *S. paradoxus* spores are placed in an environment that allows vegetative growth, they take far longer to produce buds than their *S. cerevisiae* counterparts. This may suggest that the mating pheromone has a longer time to act in *S. paradoxus*, exaggerating the difference in attractiveness between good and poor quality mates. *S. paradoxus* is still a relatively wild strain, so it may be that the ability to discriminate good and poor quality mates is more important in the wild than in the laboratory. *S. cerevisiae* has not

been subject to the same natural selection pressures. This is particularly true of long domesticated laboratory strains that are constantly put under artificial selection pressures. It is very possible that for multiple generations, researches have inadvertently been selecting for the ability to mate quickly in their strains.

6.4 Mating ecology

6.4.1 Mating propensity

This thesis included many mate choice assays that were designed to measure preferential mating in yeast. While they provided the desired results, they also raised a few questions. The assumption in yeast research is that mating usually takes place within the tetrad. Genomic studies would certainly support this theory. As such we would expect high mating propensity for the mate choice assays. This was not the case. The mate choice assay using tetrads of spores in their ascus offer potentially the most realistic formation that cells may find themselves in when reproducing sexually. Much of the time either no cells mated, or only two of the four mated.

When two cells fuse together to form a zygote, this can form a mating barrier if it bisects the remaining two cells of the tetrad. This may account for some of the single matings observed. Mate rejection could also be a potential cause of single matings. If one cell is rejected in favour of a more attractive mate, the time spent under cell cycle arrest during the courtship may mean that other potential mates may no longer be available when its first choice mate has mated. This leaves us with one

zygote and two unmated cells in many tetrads. So what of the remaining two cells? How can they find mates while still giving the impression of mating taking place within the original tetrad? If the strain is homothallic, the unmated cells can eventually change mating type after two generations and mate with their daughters. An alternate scenario is one that is not taken into account in my experiments. It is very reasonable to assume that tetrads are not isolated from each other. They are likely to be in contact, so an unmated cell (or any cell for that matter) may mate with another cell from a neighbouring tetrad. Murphy & Zeyl (2010) have showed this experimentally. They argue that as most neighbouring tetrads are liable to have descended from diploid clones, any inter-tetrad mating would still have the characteristics of intra-tetrad mating.

Obviously there is still much that is unknown about how yeast mate and under what conditions. The most obvious drawback of this is that the artificial conditions used in mate choice assays do not accurately represent the conditions in which mating actually takes place in the wild. The mate choice assays testing for mate-size preference demonstrated that yeast behaves very differently under different conditions. More realistic results would be obtained from future mate choice assays if they could be carried out under more realistic mating conditions. Similarly mate choice trials that use tetrads of spores, would probably give truer results if tetrads were not independent, but rather in contact with each other. As increased pheromone in the medium increases mating propensity, it is likely that groups of tetrads signalling together will result in more intra-tetrad matings taking place as well as inter-tetrad matings.

6.4.2 Initial growth of spores

Germination times were measured as the time taken for a spore bud. This period of time includes not only germination, but also the initial stages of mitotic growth. Given the important role that germination time initial growth seem to play on preferential mating in yeast, it is certainly a process that deserves more attention. In particular the factors that affect the rate at which germination takes place. Why do *S. paradoxus* spores germinate slower than *S. cerevisiae* spores?

6.5 Future directions

There are several competing theories that try to explain why some organisms have maintained sexual reproduction despite the large cost associated with it. While there are many arguments in favour and against many of these, it is often difficult to test them experimentally. The unique qualities of *Saccharomyces cerevisiae* make it a suitable candidate for use as a model system for such experiments. As pointed out repeatedly, their short generation time makes witnessing evolution taking place a reality. There is another important aspect of *S. cerevisiae* that makes it ideal for testing these theories. It can reproduce both asexually and sexually. This is vital, as any comparative studies testing the potential benefits of sex can be measured against an asexual population with an identical start point. The hypothetical benefits of sex could then be measured directly by subjecting both asexual and sexual populations to the various scenarios in which sex is believed to be advantageous. Increasing the number of

random mutations through mutagen treatment could test for the genetic benefits of sex (DNA repair, clearance of deleterious mutations etc). Similarly, the adaptive benefits of sex could be tested by fluctuating environmental conditions such as temperature. One theory suggests that sex allows for faster exploitation of different niches. Developing a layered medium that offered such niches could test this.

While there is already so much potential for important experiments using *Saccharomyces cerevisiae*, it is important to still find out as much as we can about its mating ecology. A more detailed understanding would allow for much more realistic experiments to be designed, while also giving context to results. Important areas that need to be addressed are the conditions and environment in which sporulation and yeast mating takes place in nature. It is evident that it is not just the mating pheromone that has a big impact on mate choice, but also germination times. More research into the outside factors that affect germination should be fruitful and provide invaluable insight into what is clearly a very important process in terms of evolution.

Sexual selection has often faced criticism (Huxley 1938, Roughgarden et al 2006). Even within sexual selection there are often contrasting views such as Fisher's runaway (1930) and Zahavi's handicap principle (1975, 1977). This makes for a very exciting and active field of research. The search to find a single unifying theory of sexual selection seems slightly misguided at times. While natural selection may be a little easier to understand as "survival of the fittest" (1864), there is no simple catch-all phrase to define sexual selection. This is due to its complexity,

and the differences in which it may act in different situations. As new species arise throughout evolution they will be subject to different selection pressures and develop vastly different life histories. “Survival of the fittest” still applies in all of these cases, but sexual selection may not. The reasons for mate choice may be different from case to case. Some may be selecting mates based on the care they provide (Bisazza & Marconato 1988), while some may prefer specific traits as indicators of heritable quality (Smith & Greig 2010). Roughgarden et al (2006) suggest that sexual selection is incorrect on the basis that offspring are a cooperative investment between males and females, and suggest an explanation of reproductive social behaviour based on cooperation between the sexes rather than competition. Although this view was also challenged (McNamara et al 2006) it did highlight an important issue. There are several factors that can affect sexual selection, some of which we may not even be aware.

Collaborations between research fields would be fruitful in understanding the basis of mate preference. Animal physiology and neuroscience may help determine how some traits are perceived and why they are favoured. In some cases we know that males are preferred as mates due to their colourful displays (Cooper & Forshaw 1977), and in some cases we have an idea of what certain attractive traits may represent (Møller & de Lope 1994), but we do not know how these traits are actually perceived to be more attractive.

Sexual selection is a powerful process that can have many implications such as changing morphology by exaggerating sexual

ornament (Cooper & Forshaw 1977) or change behaviour (Borgia et al 1985, Diamond 1988) and may also have a role to play in speciation (Knight & Turner 2004). It is a topic that often raises debate, and it is through this constant challenging of preconceptions that the field remains active. To further the field I believe more understanding of some of the fundamental processes is necessary, such as how traits are perceived by the individual, and understanding that what is actually being selected for as a consequence (heritable viability, care, sexy sons etc). Genetic and molecular studies are relatively easy to carry out using yeast. As the yeast mating pheromone provides an easy to manipulate and traceable sexual signal, it should be straightforward to find out more about what is actually being selected for at a genetic level especially if using wild strains with naturally occurring genomic variation.

6.6 Conclusion

In this thesis I have carried out experiments to further develop the yeast *Saccharomyces cerevisiae* as a model system for the study of evolutionary biology and sexual selection in particular. I have shown that the cost of sexual signalling in yeast is consistent with that of an exaggerated sexual display that may have evolved as outlined in the handicap principle (Zahavi 1975 & 1977). I have also shown that direct benefits associated with mate size can be discriminated in mate choice trials. And I have shown that the clearing of deleterious mutations through preferential mating, that has been proven to be a theoretically

stable strategy in yeast (Tazzyman in preparation), can in fact take place in practice.

I believe these experiments have demonstrated yeast's suitability as a model system for experimental evolution and the study of sexual selection through simple yet powerful experimental design. In future research I expect yeast will be used to answer some very old questions while raising new ones and igniting fresh debate.

6.7 References

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7. Appendix