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QUANTIFYING THE COMPLEXITIES OF *SACCHAROMYCES CEREVISIAE*'S ECOSYSTEM ENGINEERING VIA FERMENTATION

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Abstract. The theory of niche construction suggests that organisms may engineer environments via their activities. Despite the potential of this phenomenon being realized by Darwin, the capability of niche construction to generally unite ecological and evolutionary biology has never been empirically quantified. Here I quantify the fitness effects of *Saccharomyces cerevisiae*'s ecosystem engineering in a natural ferment in order to understand the interaction between ecological and evolutionary processes. I show that *S. cerevisiae* eventually dominates in fruit niches, where it is naturally initially rare, by modifying the environment through fermentation (the Crabtree effect) in ways which extend beyond just considering ethanol production. These data show that an additional cause of *S. cerevisiae*'s competitive advantage over the other yeasts in the community is due to the production of heat via fermentation. Even though fermentation is less energetically efficient than respiration, it seems that this trait has been selected for because its net effect provides roughly a 7% fitness advantage over the other members of the community. These data provide an elegant example of niche construction because this trait clearly modifies the environment and therefore the selection pressures to which *S. cerevisiae*, and other organisms that access the fruit resource, including humans, are exposed to.

Key words: adaptation; Crabtree effect; ecosystem engineering; ethanol tolerance; ferment; niche construction; Saccharomyces cerevisiae; *thermal profile; wine; yeast.*

Introduction

It has long been realized that organisms engineer their environments to some degree and in doing so indirectly influence other members of the community (Jones et al. 1994, 1997). However, there is debate over the extent to which niche construction or ecosystem engineering plays a role in evolution (Odling-Smee et al. 2003, Laland et al. 2004). Clearly, for a niche construction trait to have a role in evolutionary processes it must be heritable and correlated with lifetime reproductive success. However, there are no reports about the fitness effects of a niche construction trait.

Traditional wine ferments are only conducted by the microbes naturally present: this offers a window into the ecology of the community of yeasts which inhabit the fruit niche and provides a model system with which to assess the ecological and evolutionary effects of ecosystem engineering. The commonly observed community dynamics of traditional ferments are that a diversity of yeast species (~10) is found in the early ferment, but that *Saccharomyces cerevisiae* is initially very rare (Pretorius 2000, Xufre et al. 2006). As the ferment proceeds the various other hemiascomycete (non-*Saccharomyces*) species decline in frequency as *S. cerevisiae* increases until *S. cerevisiae* finally dominates and completes the ferment. Why is there such a dramatic change in community composition, and how does one species invade this niche so effectively? *S. cerevisiae* demonstrates the Crabtree effect: when sugar is above ~9 g/L, fermentation occurs even in the presence of oxygen (Piskur et al. 2006). On the face of it fermentation in the presence of oxygen is costly because it is more energetically efficient to respire the available sugar (Thomson et al. 2005).

It seems that *S. cerevisiae* is a specialist at consuming ripe fruits, and as such we should not be surprised to learn that mechanisms might have evolved in order to defend this valuable resource: ethanol production via fermentation is hypothesized to be advantageous because it acts as an agent to decrease interspecific competition and predation (ethanol is a general antimicrobial and

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acts as a deterrent to most vertebrates; Janzen 1977, Thomson et al. 2005). The decrease in non-*Saccharomyces* species correlates with the rise in ethanol, and most non-*Saccharomyces* only produce low concentrations of ethanol. While it seems to make intuitive sense that niche construction through ethanol production is the reason for *S. cerevisiae*'s increase in frequency, the few data available on the tolerance of non-*Saccharomyces* species to ethanol do not necessarily correlate with this assertion because some non-*Saccharomyces* show reasonable tolerances to high ethanol concentrations (Fleet and Gao 1988, Heard and Fleet 1988, Pina et al. 2004, Perez-Nevado et al. 2006). An alternate hypothesis suggests that it is the rate of glycolytic flux, rather than ethanol production, that selection primarily operated on (Conant and Wolfe 2007). Because *S. cerevisiae* is also the classic model used to elucidate the genetics and molecular biology of eukaryotes, and is used increasingly as a model to study general ecological and evolutionary concepts, knowledge of the forces that have shaped its genome is of general importance (Zeyl 2000, Landry et al. 2006). However, we have very little knowledge concerning the ecology of *S. cerevisiae*.

Does ethanol production provide a selective advantage for *S. cerevisiae*? I examined a community of yeasts from a traditional ferment, and quantified the fitness effect of *S. cerevisiae*'s ecosystem engineering on the members of this community. Controlled experiments on the various members of this community allowed me to test hypotheses concerning the selective pressures that promoted the change in community composition. I show that ethanol production has an effect on fitness, but it might not be the only factor of importance. Despite its inefficiency, it seems that natural selection also operated on the Crabtree effect due to the production of heat: this component of *S. cerevisiae*'s ecosystem engineering appears to have a greater impact on fitness, and therefore on the resultant community dynamics, than ethanol.

Materials and Methods

Sample site and vinification processes

Kumeu River Winery is found ~20 km northwest of Auckland on the North Island of New Zealand at 36°46′42″ S, 174°33′50″ E. I focused on the yeast associated with the juice resulting from mature Chardonnay vines (Mendoza clones). After the grapes were crushed, the juice (pH 3.24) was allowed to settle and 32 mg/L total SO_2 was added. In the winemakers' experience this amount of SO_2 is balanced to prevent oxidation (browning) of the juice and deter bacterial growth, but is at a level that does not kill the majority of yeasts that are present. Because nitrogen is one of the limiting nutrients in grape juice (with sugar at 222 g/L carbon was clearly not limiting), diammonium phosphate was added at the beginning of the ferment. The resulting juice was left to spontaneously ferment in Burgundy oak barrels (Seguin Moreau, Chagny, France) for 20 days, and O_2 was added at days 0, 7, and 8. Four barrels were tracked and 1-mL samples were taken daily aseptically throughout the 20-day ferment (each contained 225 L). These were brought back to the laboratory on ice. Ferment temperature was monitored, and the progress of the ferment was determined by the change in specific gravity.

Microbial enumeration and identification

In order to estimate the number and range of culturable yeast species, and determine the presence of *Saccharomyces* species, eightfold serial dilutions of samples were plated onto acidified malt media (5% malt extract, 0.4% lactic acid volume/volume; Johnson et al. 2004). Eighty candidate colonies were selected for molecular analyses each day. Initially the ribosomal internal transcribed spacer (ITS) 1, 5.8S rDNA, and ITS region 2 of each colony was amplified and subjected to restriction with the *Hae*III and *Hinf*I endonucleases, which produced nine different classes. The ITS and D1–D2 divergent domains of the 26S rDNA of each of the nine classes was then two-way sequenced (Kurtzman and Robnett 1998). The resulting sequences were compared to those deposited in the NCBI database using the BLASTn tool (Altschul et al. 1990). The nine species of this community identified were: *Hanseniaspora uvarum*, *Pichia fermentans*, *Pichia* sp., *Issatchenkia orientalis*, *Pichia kluyveri*, *Candida zemplinina*, *Candida railenensis*, *Issatchenkia terricola*, and *Saccharomyces cerevisiae*.

Growth rate assays

Sauvignon blanc grape juice was used in both assays and sterilized with 200 μL/L dimethyldicarbonate (DMDC) before use. YPD (1% yeast extract, 2% peptone, 2% glucose) and grape juice were supplemented to a final concentration of 0%, 3%, and 9% ethanol for a total of six environments. The growth rate of two isolates of *S. cerevisiae* and six isolates of the various non-*Saccharomyces* species (two different *H. uvarum*; *P. kluyveri*, *P. fermentans*; *I. orientalis*; *P.* sp.) was estimated by the change in optical density (OD; 660 nm) at 30°C in triplicate at 0, 19, 38, and 57 hours. The growth rates of the same two indigenous isolates of *S. cerevisiae* and four of the various non-*Saccharomyces* isolates (*Pichia* sp., *P. kluyveri*, *C. zemplinina*, *C. railenensis*) was estimated by the change in optical density (660 nm) in grape juice and YPD at five relevant temperatures (10°C, 15°C, 20°C, 25°C, and 30°C) in triplicate. Two commercial wine strains of *S. cerevisiae* (VL3 and VIN7; Bradbury et al. 2006) were also included. Measurements were taken at regular intervals until no significant change in OD was seen (this was over a 2–4 day period depending upon temperature). Controls were included in every batch to guard against contamination concerns.

The maximum change in OD was calculated for the ethanol and thermal profile data and from this an

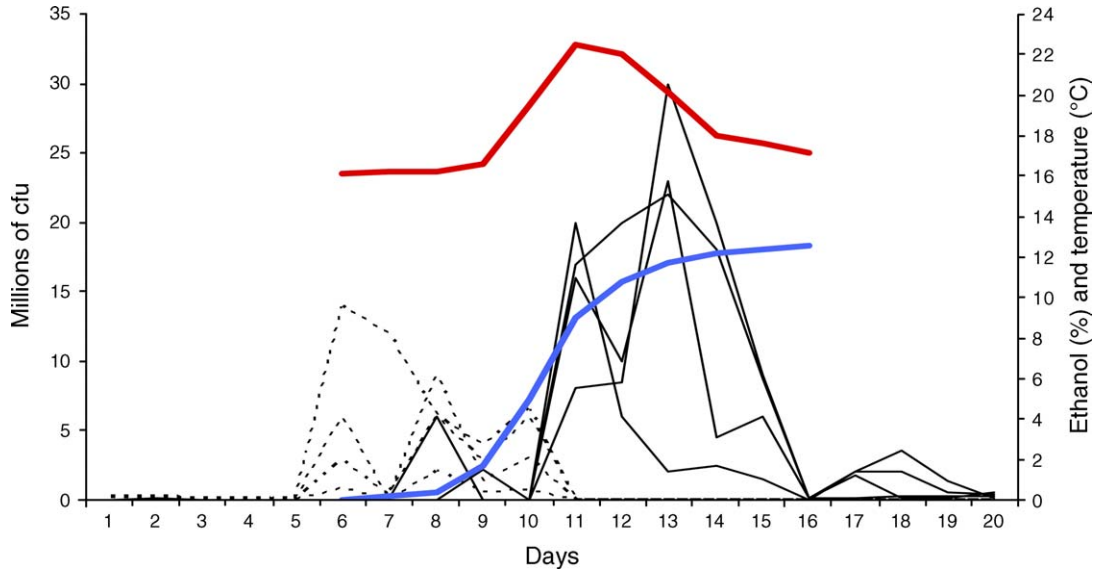

FIG. 1. The change in yeast community composition, temperature, and ethanol concentration during a traditional wine ferment. Shown is the change in population size (colony forming units, cfu) of the non-*Saccharomyces* yeasts (thin black dashed lines) and *S. cerevisiae* (thin black solid lines) in four separate barrels over 20 days of ferment. Also shown is the average change in temperature (heavy red line) and ethanol levels estimated from the change in specific gravity (heavy blue line) for these four barrels over days 6–16 of the ferment.

estimate of the rate of exponential population increase (r) (also called rate in intrinsic increase or specific growth rate [μ]) was made since $N_t = N_0 e^{rt}$, where N_0 and N_t were the initial and final OD measurements for each time period under consideration (t in hours). The mean values for the *S. cerevisiae* isolates were then compared to the mean values of the non-*Saccharomyces* isolates in order to estimate the difference in rates of exponential increase, which is denoted by m (log[fitness hour^{-1}]): m is simply rS − rN, where rS is the rate of exponential increase of *S. cerevisiae* and rN is the rate of exponential increase of non-*Saccharomyces* species. The percentage Darwinian fitness (w) is 100(exp(m) − 1). A mixed population starting with *S. cerevisiae* at frequency $p0$ and non-*Saccharomyces* at frequency $q0 = 1 - p0$ will take the following amount of time (t) to reach frequencies pt and qt, respectively (Hartl and Clark 1997):

$$t = \frac{1}{m} \ln \frac{ptq0}{qtp0}.$$

The maximum growth rate data were analyzed by various ANOVAs using the JMP package (JMP, version 5.1, SAS Institute, Cary, North Carolina, USA).

RESULTS

Nine different species of yeasts were recovered from the grape juice and ferment that resulted from this. It seems two distinct periods of microbial expansion occurred: an earlier one comprising the non-*Saccharomyces* and a later one comprising just *S. cerevisiae*. *S. cerevisiae* could not be detected on the first day of ferment; yet, by day 11 it was the dominant species. It is clear that *S. cerevisiae* was very rare initially but increased in abundance and displaced the various non-*Saccharomyces* species as it did so (Fig. 1): this concurs with yeast population dynamic observations of other traditional ferments (Pretorius 2000, Xufre et al. 2006).

Grape juice itself is a harsh medium; among other things it has a low pH of ~3.5 and it imposes an osmotic pressure because sugar is typically ~200–350 g/L (Ribereau-Gayon et al. 2006). In order to differentiate between the effects of ethanol alone, or the interaction between ethanol and the other stresses imposed by grape juice, the growth rates of the various species from the community were tested in a benign laboratory medium (YPD) and in grape juice supplemented with 0%, 3%, and 9% ethanol at 30°C. Growth rate proved to be significantly affected by media type, ethanol level, yeast species, and all possible interactions of these effects (three-way ANOVA, all main effects and possible interactions produced $P < 0.001$). The significance of these effects remained when the yeasts were grouped into *S. cerevisiae* and non-*Saccharomyces* classes, apart from the ethanol level–media interaction: the growth rates of both classes decreased as ethanol level rose. Fig. 2 shows the magnitude of these effects, and their interactions. An ANOVA shows that the difference between the growth rate of *S. cerevisiae* and the non-*Saccharomyces* in grape juice with no ethanol is highly significant ($P < 0.0001$): here *S. cerevisiae* has a 4.1% per hour fitness advantage ($m = 0.04$ h^{-1}). This advantage may not seem great, but it would allow *S. cerevisiae* to increase from 0.1% to 99.9% of a community in just 14

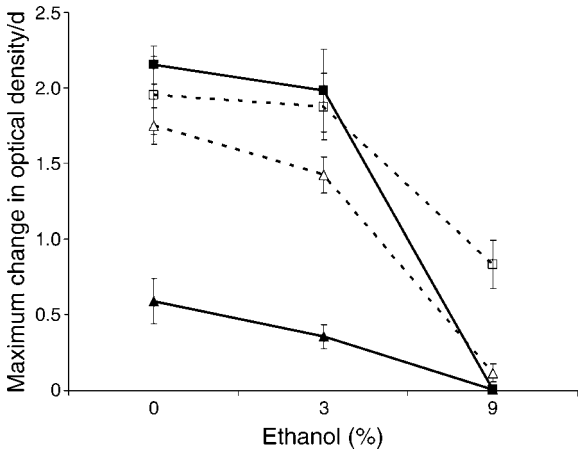

FIG. 2. The effect of environment and ethanol concentration on the growth rate of species in the yeast community. The figure shows the change (mean ± SE) in growth rate (as estimated by the maximum change in optical density [OD, absorbance at 660 nm] over 24 hours) of the non-*Saccharomyces* (triangles; mean of six species, $n = 3$ for each species) and *S. cerevisiae* (squares; $n = 6$) in YPD (1% yeast extract, 2% peptone, 2% glucose; open symbols, dashed line) and grape juice (solid symbols, solid line) supplemented with 0%, 3%, and 9% volume/volume ethanol.

days. The difference between *S. cerevisiae* and the non-*Saccharomyces* species in grape juice remains significant upon the addition of 3% ethanol (one-way ANOVA, $P < 0.0001$). In this environment *S. cerevisiae* is 6.2% per hour fitter ($m = 0.06$ h^{-1}; 10 days to go from 0.1% to 99.9%): it seems the addition of ethanol to grape juice increased *S. cerevisiae*'s competitive advantage by around one-half as much again as that seen in grape juice alone. Upon examining the data I found that there was no significant difference in growth rate between any of the species in YPD alone (ANOVA, $P = 0.5$), and that the addition of 3% ethanol to YPD did not alter this (ANOVA, $P = 0.1$). Closer examination shows that while most of the non-*Saccharomyces* species were unable to grow in YPD with 9% ethanol, one member of the community showed no significant difference in growth compared to *S. cerevisiae* ($P = 0.19$). All else being equal this isolate of *Issatchenkia terricola* is as equally competitive as *S. cerevisiae* in the presence of ethanol up to concentrations of 9%.

The nature of the environment (juice vs. YPD) had a large and significant effect on the difference in growth rate between *S. cerevisiae* and the non-*Saccharomyces* at 30°C. It seems clear that low levels of ethanol do not significantly poison the non-*Saccharomyces* yeast in this community, and that some appear tolerant of reasonably high ethanol levels. These data suggest that *S. cerevisiae*'s ecological advantage is due to the fact that it is better adapted to juice per se when compared to the non-*Saccharomyces*, and this is further compounded by the addition of ethanol. This provides evidence that the production of ethanol, and the adaptation of better growth in the presence of ethanol, is an example of evolutionary feedback from niche construction. Are these aspects the entire explanation for the change in community composition? Apart from the engineered rise in ethanol levels, there is at least one other environmental change that correlates with the decrease of the non-*Saccharomyces* and the increase in *S. cerevisiae*: temperature (see Fig. 1).

Examination of the chemistry of fermentation shows that ethanol and CO_2 are not the only products: because the reaction is exogonic, energy is also released. The theoretical energy release from the conversion of a one equimolar solution of glucose : fructose to ethanol and CO_2 is 104.43 kJ/mole (Williams 1982). Because grape juice is close to one molar glucose : fructose, this is not far off the potential energy released during ferments. It takes ~3.43 J to heat 1 mL of grape juice by 1°C. Therefore, the fermentation of 1 L of an equimolar glucose : fructose solution would liberate 104.43 kJ, and this could potentially heat grape juice up by ~30°C. Of course, this is a theoretical situation where the conversion is instantaneous, 100% efficient, and fully insulated. While these conditions are rarely met, it is clear that as well as ethanol and CO_2, fermentation produces a large amount of energy that is transferred to the environment. Indeed, the temperature of ferments can change dramatically as they proceed (Ribereau-Gayon et al. 2006). The ferments followed at Kumeu River were conducted in an air-conditioned room but still rose from ~15°C to ~25°C (see Fig. 1): this rise correlates with the increase in frequency of *S. cerevisiae*. It is not unreasonable to assume that the rise in temperature is due to the fermentative actions of *S. cerevisiae*.

Because temperature changed during the ferment, I was interested to know if this played any role in the change in community composition observed. How do the growth rates of the various members of the yeast community vary with temperature? The thermal profile of a subset of species in the community was determined in YPD and grape juice to examine the possible interaction between environment and temperature. The maximum rate of growth was significantly affected by environment (YPD or grape juice), yeast species, temperature, and by all possible interactions of these effects (as shown by a three-way ANOVA, all main effects and possible interactions produced $P < 0.0001$). Fig. 3 shows the magnitude of the interaction between environment, temperature, and yeast species. It is clear that *S. cerevisiae* has a growth rate advantage over the non-*Saccharomyces* species only in grape juice that is >20°C: here *S. cerevisiae* has an average fitness advantage of 7.3% per hour ($m = 0.07$; nine days to go from 0.1% to 99.9%). *S. cerevisiae*'s advantage is not apparent below 20°C in grape juice nor at any temperature in YPD media (there is no significant difference between the *S. cerevisiae* and non-*Saccharomyces* in YPD overall; $P = 0.06$; the marginal P value is due to the greater growth rate of the non-*Saccharomyces*

species at 30°C). One possible reason for the temperature effect in juice is due to an interaction between the low pH and temperature because these both affect cell membrane integrity: it seems that *S. cerevisiae* is better adapted to these conditions via evolutionary feedback from niche construction.

Discussion

It seems that *S. cerevisiae*'s fermenting trait (the Crabtree effect) is adaptive because, although it may be less energetically efficient, it modifies the environment to *S. cerevisiae*'s advantage in ways that extend beyond just considering the effects of ethanol. While the toxic effects of ethanol serve to poison other competing yeasts in the community, it also appears that *S. cerevisiae* is better adapted to the other stresses imposed by the juice environment at higher temperatures. I suggest an additional dimension to *S. cerevisiae*'s environmental modification: the production of heat. The rise in temperature and ethanol as a result of the Crabtree effect trait was operated on by natural selection because it increased *S. cerevisiae*'s fitness. It seems this is an adaptation that only *S. cerevisiae* possesses in this particular community: none of the other species were able to ferment the grape juice to completion (M. R. Goddard, *unpublished data*), and their lineages are all positioned before the yeast whole-genome duplication event and therefore demonstrate either an absence or significantly diminished Crabtree effect (Merico et al. 2007). These data correlate with the reports that *S. cerevisiae* dominates more rapidly in traditional ferments with higher temperature (Heard and Fleet 1988), and observations that higher temperature ferments experience less problems from non-*Saccharomyces* yeasts. It is also known that the effects of ethanol are exacerbated at higher temperatures: in keeping with the thermal hypothesis that ethanol's toxic effects on non-*Saccharomyces* yeasts have been shown to rise with increasing temperature (Heard and Fleet 1988).

How does the inference of *S. cerevisiae*'s competitive advantage in controlled laboratory situations translate into the dynamics observed in the original community? A conservative lower bound of 0.0007 (1 in ~1500) was placed on the initial frequency of *S. cerevisiae* using likelihood methods based on the binomial distribution. Taking into account both the production of heat and ethanol, the average fitness advantage that *S. cerevisiae*'s niche construction conveys in the laboratory is 6% ($m = 0.06$). This advantage could allow *S. cerevisiae* to increase from 0.1% to 99.9% of a population in 11 days. Even though this simple model does not take into account the fact that selection coefficients would change as temperature and ethanol levels change, this estimate matches the actual time it took *S. cerevisiae* to dominate the ferments monitored. The fermenting niche is undoubtedly much more complex than the few parameters examined here (for example the effects of nitrogen uptake and competing prokaryotes have not been considered), yet the change in relative selection coefficients imposed by these various environmental modifications that *S. cerevisiae* inflicts are largely sufficient to explain why *S. cerevisiae* is able to invade the fruit niche.

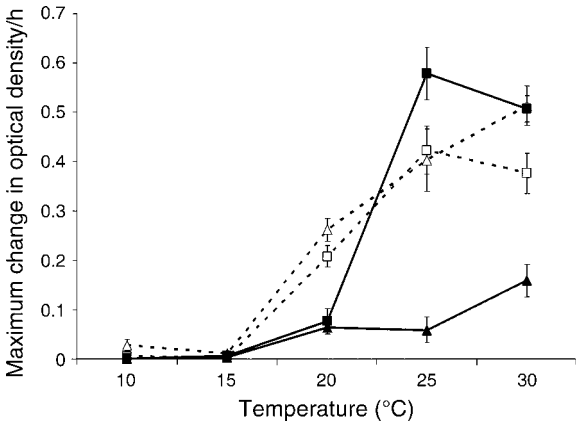

Fig. 3. The effect of environment and temperature on the growth rate of species in the yeast community. The change (mean ± SE) in growth rate (as estimated by the maximum change in optical density [OD, absorbance at 660 nm] per hour) of the non-*Saccharomyces* (triangles; four species, $n = 3$ each) and *S. cerevisiae* (squares; four isolates, $n = 3$ each) in YPD (open symbols, dashed line) and grape juice (solid symbols, solid line) across a range of temperatures.

The theory of niche construction predicts that loci other than the ones involved in construction will be subject to selection pressures as a result of the environmental modification (Odling-Smee et al. 2003). This possibly explains why at least six duplications have persisted after the yeast whole-genome duplication as these are proposed to have contributed to the Crabtree effect trait in *S. cerevisiae*, though some Crabtree effect traits are inferred to have evolved before the whole-genome duplication (Conant and Wolfe 2007). Even though *S. cerevisiae* is the best genetically studied eukaryote, ~30% of its loci have yet to be ascribed a function. Along with comparative genetic and metabolic approaches (Conant and Wolfe 2007, Merico et al. 2007), perhaps a more holistic approach, with consideration of *S. cerevisiae*'s ecology, will shed new light on the origin of its genomic architecture. Niche construction theory suggests that the evolutionary trajectory of other members of the community, which access the niche, will potentially be altered. This is possibly demonstrated by the fact that some of the non-*Saccharomyces* yeasts are able to tolerate much higher levels of ethanol than they make themselves (Hansen et al. 2001, Perez-Nevado et al. 2006). More distant effects are the selection pressures imposed on other organisms as a result of *S. cerevisiae*'s niche construction. For example, *Drosophila* are cued to locate ripe fruits through ethanol plumes (Parsons 1980), as are higher primates, which are predominantly frugivorous (Dudley 2004). The Crabtree effect has likely exposed the human lineage to selection pressures that have affected our

evolutionary trajectories: among other things, the consumption of ethanol has led to an increased resistance to its effects (Oota et al. 2007). Perhaps the most striking interpretation is that *S. cerevisiae*'s ecosystem engineering had a hand in the origins of civilization: it has been suggested that the planting and storage of grain for the production of bread and beer catalyzed the move from a nomadic to a static mode of life, which allowed civilized activities to flourish (Mortimer 2000, Salamini et al. 2002, Standage 2007).

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