Long-Term Evolution of the Complex Eukaryote *Tetrahymena thermophila*

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ABSTRACT

Evolutionary biologists have long sought to understand what factors affect the rate and repeatability of adaptive outcomes. To better understand the role of temperature in determining adaptive trajectories, we evolved populations of different genotypes, including populations that just had sex and populations that hadn't, of the ciliate *Tetrahymena thermophila* at two temperatures and followed changes in growth rate over 1000s of generations. As expected, growth rate increased with a decelerating rate for all populations; however, there were differences in the patterns of evolution at warmer and colder temperatures. Initial differences in growth rates between the genotypes decreased as evolution proceeded at both temperatures, but this convergence was quicker at the higher temperature. Likewise, we found greater repeatability of evolution among replicate populations of the same genotype at the higher temperature. We also found no evidence of trade-offs in fitness between temperatures, but did observe asymmetry in the correlated responses, whereby evolution in a warmer temperature increases growth rate at the lower temperature significantly more than the reverse. We also find the populations founded from a single newly produced sexual progeny are more evolvable than their parents who have not had sex in many generations. These results demonstrate the importance of genotype and the environment in determining evolutionary trajectories.

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Chapter I

Introduction

Understanding the diversity of life and how it came to be has been a difficult task for biologists. This task is made more difficult because of the fact that we are not casual bystanders but part of the diversity. There is a long anthropic tradition of viewing humanity as the pinnacle of life, e.g. scala naturae (Aristotle). Indeed, to this day many people continue to view life and biology, unlike physics or chemistry, through an utterly unscientific lens because they are part of it. People desire a solid explanation of where they came from and it is only very recently in the history of humanity that we have the ability to address the problem in a more systematic way.

Darwin and others revolutionized this picture allowing questions to be asked that would have previously been inconceivable. The era of molecular biology has opened a goldmine of genetic data that we have used to patch together the past. However, without a time machine the detail in our story is limited to what we can infer from archeological and genetic data. One way to more completely understand the evolutionary process is through the use of experimental evolution. Experimental evolution is a tool that allows scientists to “replay the tape of life” (Lobkovsky and Koonin 2012; Blount et al. 2018) in order to test hypotheses about the mechanisms that drive evolutionary change (Blount et al. 2008). In many cases a subsample of the population can be frozen at various timepoints to be thawed at a later date creating a living fossil record of evolution from which evolution can be replayed. These experiments allow researchers ask questions about the roles of history, chance, and

selection in determining evolutionary outcomes (Travisano et al. 1995; Rebolleda-Gómez and Travisano 2019).

The primary method of experimental evolution involves allowing replicate populations to evolve in the controlled setting of the laboratory (Lenski and Burnham 2018). In this way changes in phenotypes and genotypes can be tracked in real time with knowledge of the demographics and environmental conditions of the populations. Experimental evolution has been used to test a variety of questions in diverse organisms. For example, several experiments utilizing *Drosophila melanogaster* have found signatures of parallel evolution the genomes of flies from different replicate populations (Burke et al. 2010; Graves et al. 2017) in some cases this parallelism was beyond what was predicted by known models (Phillips et al. 2016). Interestingly, no hard sweeps were detected in any of these experiments. *Caenorhabditis elegans* has also been a useful animal system in which many evolution experiments have been performed (Gray and Cutter 2014). These experiments have focused on a variety of topics including coevolution (Schulte et al. 2010), the effects of mutation (Denver et al. 2012), mating systems (Manoel et al. 2007), and the evolution of life history traits (Walker et al. 2000).

Microbial systems are particularly amenable to experimental evolution because of their short generation time and ease of manipulation in the lab. The most famous and longest running microbial long-term evolution experiment was started by Richard Lenski in 1988 and continues to this day. To date the 12 replicate populations have

been evolving for ~70,000 generations and been the subject of numerous investigations and papers. These studies have revealed the emergence of complex ecological interactions in many of the populations (Good et al. 2017), the fixation of hypermutable genotypes in some of the populations (Couce et al. 2017), and the evolution of a citrate utilizing mutant in one of the populations (Blount et al. 2008). One of the most surprising results from this experiment is the fact that fitness is still increasing even after 50,000 generations of evolution (Lenski et al. 2015). This was surprising because theory suggested beneficial mutations would have been exhausted and fitness would have plateaued well before 50,000 generations. This result helped revealed the importance of epistasis in the evolutionary process and in part lead to research showing non-additive genic interactions are in fact common (Kuzmin et al. 2018).

While much attention has been paid to mapping complex networks leading from genotype to phenotype and finally to fitness (Venkataram et al. 2016; Costanzo et al. 2019), there is still much to be learned about how fitness and fitness related parameters change as evolution progresses. I refer to the rate at which fitness changes as evolvability and the inverse of the variation that arises between populations as the repeatability of evolution. These are two of the most important evolutionary parameters which can inform us of a population's likely fitness trajectory and how deterministically that trajectory is followed.

Many papers have been written over the years concerning some notion of evolvability. Work describing differences in how populations respond to selection dates to the 1930's (Fisher 1930) but the first use of the term evolvability is generally attributed to Dawkins who used it to describe the ability of a lineage to spawn evolutionary radiations in 1989 (Dawkins 1989). Since then it has been used in variety of ways (Alberch 1991; Houle 1992; Pigliucci 2008; Woods et al. 2011). Houle's quantitative genetic approach essential treats evolvability as heritability but ignores the generation of new variation which is vital for long-term evolution. Other approaches attempt to define evolvability by how quickly a trait can change due to standing genetic variation and the generation of new variation. More recent approaches attempt to simplify the definition of evolvability by considering how only a single trait, fitness, can change over time (Woods et al. 2011). These methods quantify evolvability by comparing the change in fitness that occurs over some number of generations.

A lot of work has also focused on the relationship between biological robustness and evolvability (Gerhart and Kirschner 2007; Wagner 2008; Masel and Trotter 2010). Robustness refers to the ability of phenotypes to stay the same environmental or genetic perturbations in the form of mutations. While it might appear that genetic robustness would limit evolvability if phenotypes do not change easily, it could also increase evolvability by allowing more variation to accumulate in a population (Wagner 2008). This variation may not be beneficial to any single individual in the population but it allows the population to spread out over more of the genotype

landscape potentially facilitating future adaptation (Masel and Trotter 2010). The ability of populations to harbor this type of cryptic genetic variation is referred to as evolutionary capacitance (Bergman and Siegal 2003) . Several mechanism have been proposed as evolutionary capacitors including the role of the yeast Hsp90 in releasing phenotypic variation in response to stress and the [PSI +] yeast prion causing read-through errors during translation (Masel and Bergman 2003; Ruden et al. 2003; Cowen and Lindquist 2005; Chen et al. 2012). Similar increases in evolvability are also thought to result from stress-induced mutagenesis and other forms of bet-hedging particularly in microbial populations of large size (Bjedov et al. 2003; Foster 2007; Galhardo et al. 2007; Veening et al. 2008; Beaumont et al. 2009; Maxwell and Magwene 2017; Carey et al. 2018).

The evolvability and repeatability of evolving replicate populations is dependent on the size of the evolving populations, the ancestral genotype of the population, and the environment in which evolution occurs (Bailey et al. 2015; Bauer and Gokhale 2015; Jerison et al. 2017). Experimental evolution allows us to control for the size of populations to ask questions about how genotype and environment can affect evolutionary trajectories.

Replicate populations could be more evolvable or repeatable in some environments than in others (Bailey et al. 2015). Likewise, replicate populations of certain genotypes could be more evolvable than replicate populations of other genotypes (Jerison et al. 2017). However, the extent to which evolvability is itself shaped by the

forces of natural selection is an open question (Pigliucci 2008). The variety of ways that different organisms generate variation and then sort it into individuals and populations (e.g., sex vs. asex) may provide some clue to how and when evolvability can be shaped by selection. The manner in which hereditary information gets packaged and inherited from one generation to the next (e.g., sex vs. asex, mitosis vs. amitosis, circular vs. linear chromosomes, number of chromosomes and ploidy, or chromosomal modifications) can have profound implications on the long-term evolvability of that information (Selmecki et al. 2015; Lachapelle and Colegrave 2017). If evolvability and the manner in which hereditary information is packaged and inherited within populations and thru generations is itself evolving adaptively, we might expect the generation of variation to be specially tuned to the specific population structure, demography, and environmental heterogeneity faced by the species (Hinton and Nowlan 1987; Watson and Szathmáry 2016).

Natural selection acts on variation within populations to optimize fitness over many generations. This process is made less repeatable by the stochastic nature of evolutionary innovations. Mutations are random and will not happen concurrently in all populations (Luria and Delbrück 1943). At the same time, which genotypes are present in a population and the environmental context could bias the set of mutations that are likely to fix, making evolution more repeatable by constraining it to a particular trajectory (Lobkovsky and Koonin 2012; Bailey et al. 2015). We know that the repeatability of such evolutionary trajectories is in-part determined by the

stochastic nature in which new mutations arise and are then fixed in populations. It is less clear how, if at all, genotype and environment affect this process.

The extent to which the fitness of replicate populations evolve in parallel, i.e., the repeatability of evolution, is determined by the population size, the rate of mutation and distribution of their effects, and the underlying “ruggedness” of the fitness landscape, which in turn are determined by the genotype and environment (Bank et al. 2016; Lachapelle and Colegrave 2017). Genotypes could differ in their rate of mutation and distribution of their effects and in the underlying “ruggedness” of the fitness landscape. This could result in differences in the repeatability of their fitness trajectories with replicate populations founded from certain genotypes diverging more than other genotypes (Weinreich et al. 2005; Bauer and Gokhale 2015; Van Dijk et al. 2017). Genotype could also constrain the adaptive potential of an evolving population if the population gets stuck at a local fitness optimum (Jerison et al. 2017). This could happen to replicate populations during an evolution experiment or could apply to one genotype at the start of an experiment. On a smooth fitness landscape we would expect two populations with different starting finesses to converge toward the same fitness optimum but in reality epistatic interactions can prevent this from happening (Weinreich et al. 2005). How populations can bridge these valleys in the fitness landscape and escape genetically imposed constraints has been a longstanding challenge for evolutionary biologists. One simple answer appears to be environmental heterogeneity (Steinberg and Ostermeier 2016). When the environment changes mutational paths that once would have been impossible could

open up creating ridges where valleys once existed in the fitness landscape. The correlated effects of how evolution in one environment affects fitness in another are particularly important in this light. Like genotype, the environment could also affect the rate of mutation and distribution of their effects and/or the degree of epistasis and number of adaptive peaks available to the starting population, consequently affecting the repeatability of evolution (Bailey et al. 2015).

Here I utilize long-term experimental evolution of the single-celled eukaryote *Tetrahymena thermophila* to ask how environment and genotype affect evolutionary trajectories and their repeatability paying particular attention to any larger trends that emerge. *Tetrahymena thermophila* is a free-living, single-celled, facultatively sexual ciliate with two nuclei: a silent germline micronucleus (MIC) and a transcriptionally active somatic macronucleus (MAC). The MAC gets destroyed after sex and a new one gets created from the mitotic product of the new zygotic MIC. The MAC contains ~45 copies of every chromosome (~225) and divides amitotically (Doerder et al. 1992). This means that the chromosomes do not line up and segregate as they do during normal metaphase and anaphase. Instead, the content of the genome is divided randomly between the daughter cells. While they are able to maintain approximately 45 copies of each chromosome over time they will lose one or the other parental allele until the entire genome, except for *de novo* mutations, is homozygous for one parental allele or the other. This process is known as phenotypic assortment (Merriam and Bruns 1988). Some of the oldest known asexual eukaryotic lineages are in *Tetrahymena* and a large portion of natural isolates are

amicronucleate and therefore asexual (Doerder 2014). The unique genome architecture may allow for the success of asexuals by providing some of the benefits of sex in its absence (Zhang et. al., pers. comm.) and could impact evolutionary dynamics in a number of ways. By comparing the evolutionary trajectories of replicate populations of different genotypes evolving under different environments I can assess the role of environment and genotype in determining evolutionary outcomes. We can also compare our data to similar data collected in other species in the hopes of identifying differences in the evolutionary dynamics between the major groups of organisms (e.g., eukaryotes vs. prokaryotes).

In Chapter II, I focus on the role of temperature in determining the repeatability of evolution. I allowed populations of *T. thermophila* to evolve at high and low temperatures and measured their fitness over 4000 generations. I utilized starting genotypes with different levels of fitness so I could also assess the role of temperature in promoting convergence among genotypes. I also measured the correlated response in fitness at the other temperature and looked at the symmetry of these responses as evolution progressed.

In Chapter III, I focus on the unique genome architecture of *Tetrahymena* and its impact on evolvability. Understanding the mechanisms that generate genetic variation, and thus contribute to the process of adaptation, is a major goal of evolutionary biology. *Tetrahymena thermophila* is a ciliate with an unusual genetic feature that results in a phenomenon called phenotypic assortment, which may allow for an increase in the

amount of genetic variation following sex, thereby increasing its evolvability. To test this hypothesis, I compared the rate of adaptation in *T. thermophila* populations that had just had sex to those that hadn't in many generations. The populations that were founded by a single sexual progeny adapted more quickly. This suggests that the additional genetic variation generated by phenotypic assortment can increase the rate of adaptation following sex.

Chapter II

Temperature affects the repeatability of evolution in the microbial eukaryote *Tetrahymena thermophila*

Introduction

The evolutionary trajectories of both natural and experimental populations are often remarkably similar to each other (Lenski and Travisano 1994; Colosimo et al. 2005; Woods et al. 2006; Conte et al. 2012; Nosil et al. 2018). However, there can also be substantial differences in the trajectories of initially identical experimental populations (Blount et al. 2008) and natural populations (Dieckmann and Doebeli 1999; McKinnon and Rundle 2002; Barluenga et al. 2006). While these types of studies have provided valuable insight into the repeatability of evolutionary trajectories, we still lack a comprehensive understanding of what conditions are likely to constrain trajectories from diverging due to stochastic forces, and thus contribute to the repeatability of evolution.

Previous work has demonstrated that temperature can fundamentally alter evolutionary outcomes, for example by increasing biological diversity at lower latitudes (Roy et al. 2002; Gillooly et al. 2004; Allen et al. 2006). One purported explanation for the effect of temperature is that mutation rates are different at different temperatures. However, empirical results are mixed, with some results showing higher mutation rates at higher temperatures, others lower rates at higher temperatures, and yet others are inconclusive (Faberge and Beale 1942; Kiritani 1959; Lindgren 1972). The “hotter is better” hypothesis predicts that warm-adapted populations will have higher maximum performance than their cold-adapted counterparts because of the evolution of greater robustness due to the inherently higher rates of biochemical reactions at higher temperatures (Huey and Bennett 1987; Angilletta et al. 2010). Evidence from comparative and experimental populations largely supports this hypothesis (e.g., Knies et al. 2009), however some results are mixed (reviewed in Angilletta et al. 2010). Evidence from lab evolved *Escherichia coli* shows that more adaptation occurs in hotter temperatures and that trade-offs are found at hotter temperatures when evolution occurs at colder temperatures but not vice-versa (Bennett and Lenski 1993; Mongold et al. 1996). Later work suggested that while the genetic changes underlying temperature adaptation were temperature specific, these mutations were also beneficial across all temperatures (Deatherage et al. 2017), demonstrating that observed trade-offs are not due to antagonistic pleiotropy, at least for the most common mutations observed. These results demonstrate that temperature fundamentally affects adaptive

outcomes, yet it remains unknown whether the temperature at which a population evolves will also affect the repeatability of adaptive trajectories.

To assess how temperature affects the repeatability of evolution, we performed a long-term evolution experiment using the microbial eukaryote, *Tetrahymena thermophila*. *T. thermophila* is useful as a model system due to its complex life history and development, and its ease of growth and tractability in lab (Nanney 1974; Merriam and Bruns 1988; Prescott 1994). The short generation time and small size mean that large populations can be evolved over many generations in lab, and population size and growth rate are easily monitored. In addition, in contrast to most other microbes in which experimental evolution is regularly performed, it has a complex life history and genome structure (Nanney 1974; Merriam and Bruns 1988), allowing us to test whether the general patterns found in other microbes also apply to ciliates.

T. thermophila, like all ciliates, is notable for its genome structure. Two types of nuclei are maintained in each cell. The germline micronucleus (MIC) is diploid and transcriptionally silent during growth and asexual reproduction, while the somatic macronucleus (MAC) is 45-ploid and transcriptionally active, meaning it gives rise to the phenotype of the cell (Merriam and Bruns 1988). Ciliates are facultatively sexual, mostly reproducing asexually, but occasionally undergoing conjugative sex with cells of a different mating type (Nanney 1974). In our experiment, populations contained a

single mating type, effectively preventing sex. Thus, only mutations that occurred in the MAC were subject to selection and captured in our fitness assays.

Two features of the *T. thermophila* genome may potentially impact the patterns of adaptive evolution. First, the polyploid MAC divides by amitosis, a process that results in the random distribution of alleles among daughter cells. Unlike with division by mitosis, amitosis results in allelic variation among asexual progeny (Doerder et al. 1992), which generates higher levels of genetic variation and potentially increases the rate of evolution. Second, *Tetrahymena* has an exceptionally low base-substitution mutation rate (Long et al. 2016), which has the potential to slow the rate of adaptation. However, the deleterious mutation rate is comparable to other species (Long et al. 2013), so the potential effect of mutation rate is currently unclear.

In this study, we conducted a long-term evolution experiment to determine how temperature affects repeatability of evolution in a ciliate. We evolved populations of different genotypes of *T. thermophila* in two different temperatures and monitored the fitness trajectories of replicate populations. To assess the effects of temperature on the dynamics of evolutionary trajectories, we ask: 1) Does evolution temperature effect the future convergence or continued divergence of initial historical differences between genotypes, 2) Does evolution temperature affect the repeatability of fitness trajectories, and 3) How temperature-specific are adaptations, i.e., are there trade-offs or other correlated responses between temperatures?

We find that populations evolved at the higher temperature tended to have higher fitness than their colder-evolved counterparts. The higher evolution temperature also led to faster convergence among populations started from different genotypes, and less divergence among replicate populations of a single starting genotype, indicating that evolution at a higher temperature does indeed result in more repeatable evolution. Finally, we found no indication of trade-offs, but rather an asymmetry in the correlated responses, whereby evolution at the higher temperature increases fitness at the lower temperature more than the reverse, possibly indicating greater environmental specificity of adaptations at the lower temperature.

Methods

Summary

We allowed 12 populations to evolve at 24 ºC and 12 populations to evolve at 37 ºC. Each set of 12 populations consisted of four replicate populations of three initial genotypes: two independent natural isolates and a hybrid progeny of these two isolates. Throughout the course of 4000 generations of evolution, we measured growth rate at 24 ºC and at for each population.

Strains and initial cross

Natural isolates of *T. thermophila*, designated 19617-1 (Tetrahymena Stock Center ID SD03089) and 19625-2 (Doerder 2019), were thawed from frozen stocks, inoculated into 5.5 mL of the nutrient rich medium SSP (Gorovsky et al. 1975) in a 50 mL conical tube, and incubated at 30 ºC with mixing for two days. These cultures

were maintained as the parental lines. Eight populations were established for each genotype in 10 mL cultures in SSP. Four of these were maintained at 24 ºC and four at 37 ºC. These populations were designated by genotype (19617-1 or 19625-2 herein referred to as A and B, respectively) – replicate (1-4) – and evolution temperature (24 ºC or 37 ºC), e.g. A-1-37.

To generate the hybrid genotype from these strains, a conical tube of each parental genotype was centrifuged and the supernatant was poured off before the cells were re-suspended in 10 μM Tris buffer (Bruns and Brussard 1974). After mixing at 30 ºC in Tris for two days to starve the cells and induce sexual competence, 1 mL of each starved parental population and an additional 1 ml of 10 μM Tris buffer were added to one well in a six-well plate and placed back in the 30 ºC incubator. The next morning (~12 hours later) the plate was checked for pairs and put back in the incubator for an additional 4 hours to allow progression of conjugation. Individual mating pairs were isolated under a microscope using a 2 μL- micropipette and placed in 180 μL of SSP in one well of a 96-well plate. The plate was then incubated for 48 hours after which time a single cell was isolated from each well and re-cultured into 180 μL of fresh SSP in a new well. After another 48 hours at 30 ºC four individual cells were isolated from one of the wells, into new wells with SSP, one for each of the replicate populations, and incubated at 30 ºC for 48 hours. Each of the four 180 μL cultures was then split in two with each half being added to a separate 50 mL conical tube containing 10 mL of SSP, one designated for evolution at 37 ºC and the other at 24

ºC. These eight cultures are the starting hybrid populations and are designated as A×B (19625×19617) – replicate (1-4) – evolution temperature.

This provided us with a total of 24 populations consisting of three genotypes, two parental and one hybrid, half of which were evolved at 24 ºC and half at 37 ºC with four replicate populations of each genotype per treatment.

Transfer regime

Approximately 25,000 cells (~90 μL) from each 37 ºC culture and 60,000 cells (~1 mL) from each 24 ºC culture were transferred to 10 mL of fresh SSP daily. Transfer volumes were adjusted as needed to maintain the same starting culture density at each transfer. On average, the 37 ºC evolved populations achieved ~6.8 generations per day and the 24 ºC populations achieved ~3.5 generations per day. This means that 37 ºC evolved populations experienced a wider range of densities (~2,500 cells/mL – ~275,000 cells/mL) than 24 ºC evolved populations (~6,000 cells/ mL – ~60,000 cells/mL), starting with a lower density and ending at a higher density. We estimate the effective population size to be approximately 100,000 cells for each evolved environment by calculating the harmonic mean of the population size at each discrete generation (Karlin 1968). To date, the 37 ºC populations have undergone ~9000 generations of evolution and the 24 ºC populations have undergone ~4000 generations of evolution. Here we describe the changes in growth rate over the first 4000 generations of evolution at each temperature.

Growth curves and analysis

As evolution progressed, growth rates of each population were measured at both 37 ºC and at 24 ºC, i.e. at both the temperature at which they evolved and the alternate temperature. Growth rate was measured for either temperature on average every 10-30 generations. Growth rate was measured by inoculating ~500 – 1000 cells into one well of a 96-well plate and measuring the optical density (OD) at 650 nm in a micro-plate reader every 5 minutes over the course of 24 – 48 hours for 37 ºC assays and 48 – 72 hours for 24 ºC assays (see below for validation of use of OD_{650} as a proxy for cell density). The maximum growth rate was then estimated for each well by fitting a linear regression to the steepest part of the growth curve, estimating the maximum doublings per hour (h^{-1}) (Wang et al. 2012; Long et al. 2013). 3 – 4 replicates of all populations were measured on a plate at each time point. ~375 plates containing 37 ºC evolved populations and ~625 plates containing 24 ºC evolved populations were run providing approximately 500 – 1000 growth curves at either temperature per population over the 4000 generations analyzed here.

Validation of optical density as proxy for cell density

To validate that OD accurately measures cell density over a range of densities, cells from cultures growing on the micro-plate reader were counted under the microscope at several points during the growth cycle. 3-4 replicate wells were inoculated and the plate was run on the micro-plate reader at 37 ºC. Every two to three hours, 5 μL of culture was removed and at least 200 cells were counted to estimate cell density. The cells were diluted as needed and then counted in 10 μL droplets containing

approximately 40 cells. This process was independently repeated two times. The cell density measured by counting was tested for correlation with the OD measured by the micro-plate reader at each time point, and OD was found to be a good indicator of cell density (Pearson's correlation coefficient = 0.9602; Fig. S1).

Correlation of competitive fitness and growth rate

We measured the competitive fitness of a subset of the evolved lineages after ~1000 or ~3500 (for populations evolved at 24 °C or 37 °C) generations and compared this fitness metric to our measurements of growth rate. Competitive fitness was measured in replicate by competing a GFP labeled strain (Cui et al. 2006) against the experimental strain. The two strains were mixed in approximately 1:1 ratios and the density of both strains was determined using a flow-cytometer. The culture was allowed to grow overnight at room temperature after which time the flow-cytometer was used again to measure the ratio of the two strains. Competitive fitness was calculated by dividing the natural log of the ratio of the final population density to the initial population density of one strain by the natural log of the ratio of the final population density to the initial population density of the other strain (Wiser and Lenski 2015). Competitive fitness estimates correlated with our growth rate estimates (Pearson's correlation coefficient = 0.7999; Fig. S2) indicating that growth rate is a good proxy for fitness.

Data analysis

~36,000 growth curves were collected from all populations over the first 4000 generations of evolution. This provided us with ~1500 growth rate estimates per population over this period, approximately half at either temperature. This growth rate data was binned into 250-generation intervals (generation 0 = 0-125, generation 250 = 125-375, generation 500 = 375-625, etc.) and the mean growth rate at both temperatures for each population was calculated. For each population the bin with the highest growth rate for either temperature was identified and the absolute and percent increase in growth rate was calculated from this. An ANOVA testing the effects of genotype, assay temperature, and evolution temperature was performed on these data (Tables S2 and S3). For each ANOVA the residuals were checked for heteroscedasticity visually and by regression analysis and none was detected. ANOVAs were also performed separately on the 48 data points (24 populations x 2 assay temperatures) in each bin to test for the effect of assay temperature, evolved temperature, genotype, and their interactions as evolution progressed (Tables S5, S6, and S7).

To test for significant differences among populations evolved from a single ancestor nested ANOVAs were performed on the binned data. This analysis (Table S8 and S9) tested the effects of replicate population treated as a random effect and nested within genotype, genotype, assay temperature, and the interaction between genotype and assay temperature on the mean growth rate of each population per plate (*mean growth rate/plate ~ genotype, replicate population[genotype]&Random, assay*

*temperature, genotype*assay temperature)*. To test for differences in the variance among replicate populations between evolution temperatures, ANOVAs were performed separately for each evolution temperature. This analysis (Fig. 2.4) tested for effects of replicate population treated as a random effect and nested within genotype and genotype on the mean growth rate of each population per plate in the evolution environment. From this, variance components attributable to replicate population were computed to assess the amount of variation that results from differences among replicate populations; the inverse of this was our measure of repeatability. The same analysis was performed without nesting replicate population in genotype to assess the total variance among all populations as evolution progressed (Fig. 2.5). At each binned time point, Levene's tests were performed to assess the significance of differences between evolution temperatures in the variation in growth rate generated by differences both among replicate populations of a single starting genotype and among all populations regardless of genotype.

Results

Growth rate increases with a decelerating rate of return

The trajectories of evolving laboratory populations often follow a pattern of a decelerating rate of return, characterized by larger fitness increases early in the experiment, followed by incrementally smaller increases in subsequent generations (Couce and Tenaillon 2015; Schoustra et al. 2016; Wünsche et al. 2017). In our experiment this same pattern is seen at both temperatures (Fig. 2.1) and in all three genotypes when analyzed separately (Fig. 2.2). The mean absolute increase (i.e.,

maximum mean population growth rate in a 250-generation bin minus the growth rate of the ancestor of that population) and the mean relative increase (i.e., (absolute increase/ancestral growth rate) x 100) in growth rate are reported for each combination of genotype, evolution temperature, and assay temperature in Tables S1a and S1b.

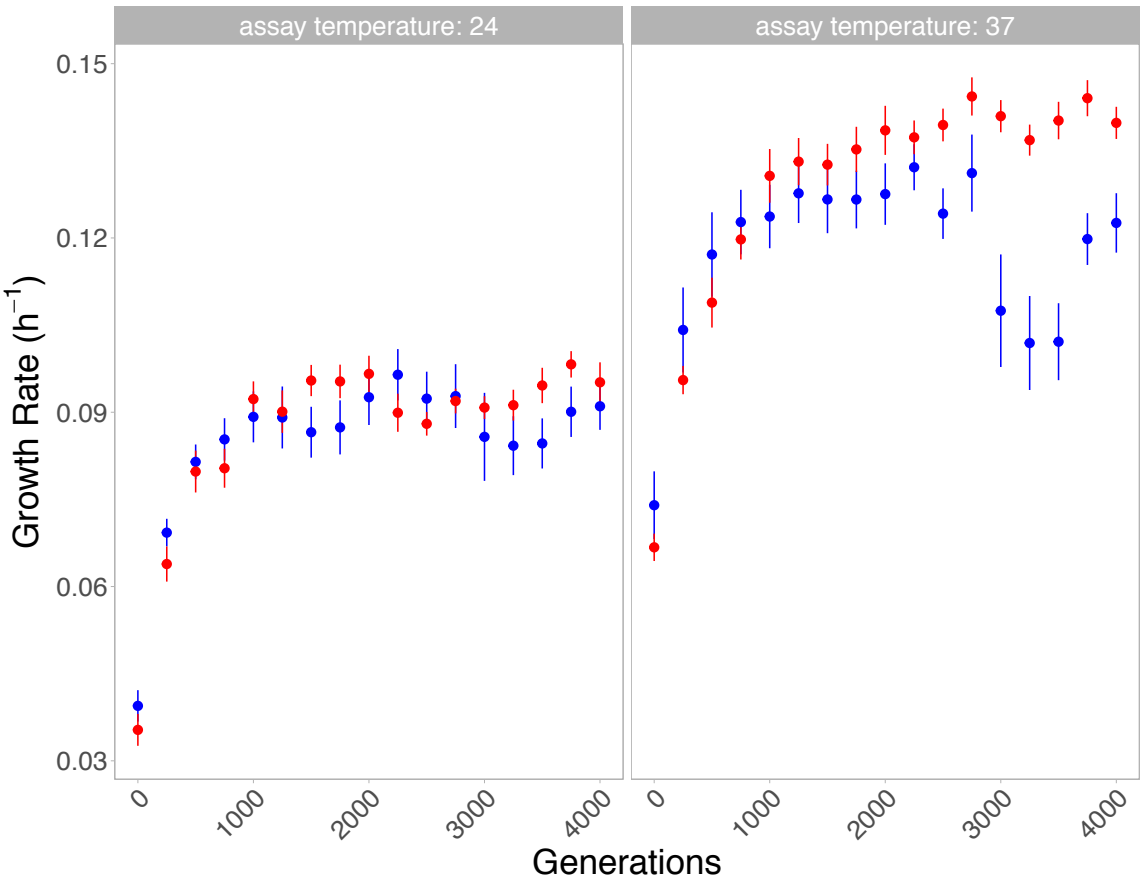

Figure 2.1. Overall pattern of evolution across all populations assayed at 24 ºC and 37 ºC. Mean growth rate and 95% confidence intervals of populations evolved at 24 ºC (blue) and 37 ºC (red) when assayed at 24 ºC (left panel) and 37 ºC (right panel) are shown over 4000 generations. Data are binned into 250 generation intervals, with the first bin containing generations 0-125.

Previous experiments have also shown that populations founded by initially slower growing genotypes tend to increase more in growth rate over the course of an experiment than those founded by initially faster growing genotypes (Jerison et al.

2017; Wünsche et al. 2017). We found a qualitatively similar result whereby genotype had a significant effect on the absolute increase (ANOVA: F (2,38) = 4.48, P = 0.0179; Table S2) and the relative increase (ANOVA: F (2,38) = 192.39, P < 0.0001; Table S3) in growth rate, with populations founded by the slowest growing genotype (A) increasing most for all four combinations of evolution temperature and assay temperature. However, due to the small number of genotypes (3) used in this experiment we cannot definitively say this effect is due to the initially lower starting growth rate of genotype A. Overall, these results indicate that experimental evolution in the ciliate *T. thermophila* does not fundamentally differ from other taxa.

However, unlike the long-term evolved *E. coli* lines which continue to increase in fitness even after 60,000 generations (Lenski et al. 2015), we find no significant change in mean growth rate among populations over the most recent 1000 generations of evolution; in fact our estimate of mean growth rate drops slightly from 0.1151 divisions per hour (h^{-1}) at 2750 generations to 0.1130 h^{-1} at 4000 generations. Additionally, for 22 out of the 24 populations a hyperbolic model yields a substantially better fit, with lower SSE values, than a power law model with the same number of parameters (k = 2; AIC evidence ratio ranged from 6.39×10^{3} -3.02×10^{14}; Table S4). This suggests that the populations may have reached growth rate optima upon which

further improvement is unlikely. However, given the limited number of generations and smaller population sizes, we are cautious in interpreting this result as further evolution could lead to increases in growth rate altering our model fits. It is also important to consider that fitness could be increasing in ways that are not captured by our growth curves so that growth rate may have plateaued while fitness is still being optimized in other ways e.g., increase in carrying-capacity or decrease in lag-time (Li et al. 2018).

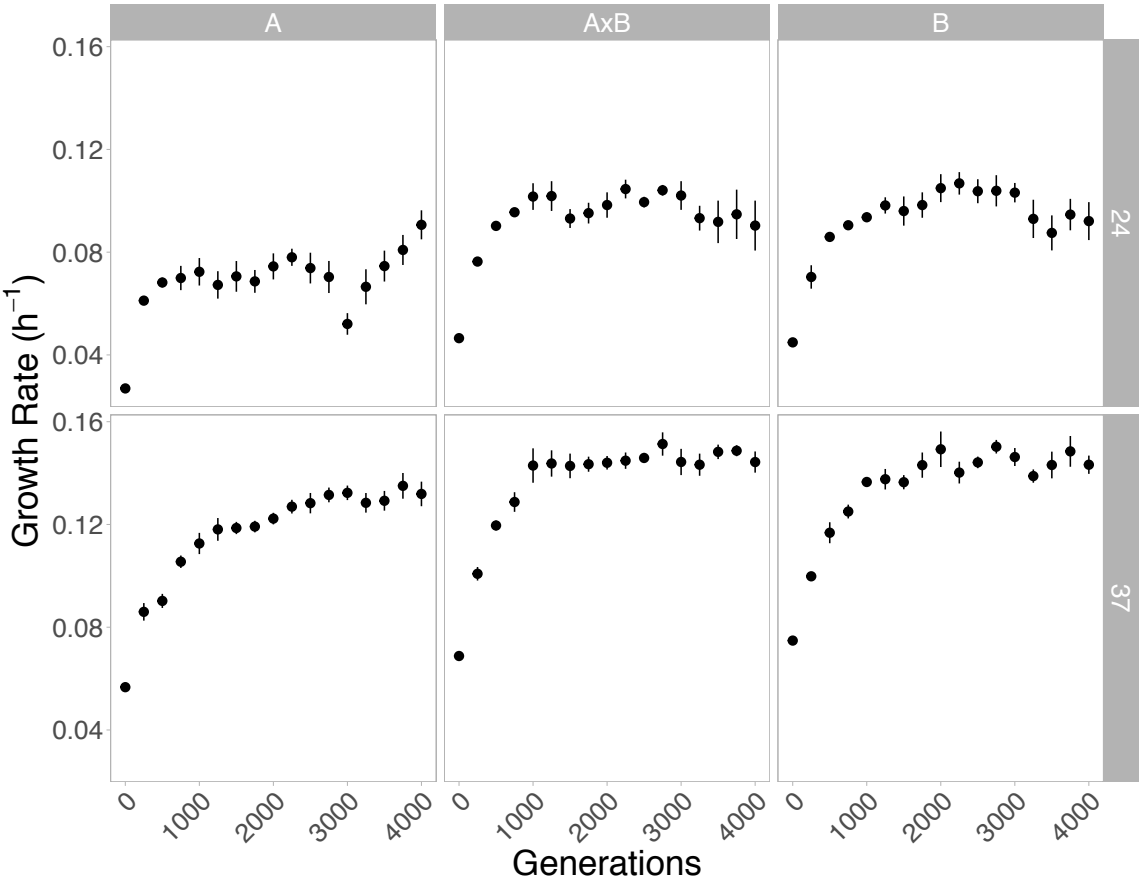

Figure 2.2. Fitness trajectories of each genotype assayed in their evolved temperature (correlated response at alternative temperature not shown). Mean growth rate and 95% confidence intervals of four replicate populations for each genotype are shown over 4000 generations. The top panels show populations evolved and assayed at 24 ºC and the bottom shows populations evolved and assayed at 37 ºC. Data are binned as in Fig. 1.

Evolution at a higher temperature results in faster convergence among genotypes

At the start of the experiment there was a significant difference in growth rate between one of the genotypes and the other two (ANOVA: F (2,38) = 189.38 P < 0.0001; Table S5). This was true whether populations were assayed at 37 ºC or 24 ºC (Wilcoxon tests; Fig. 2.3). Specifically, one of the parental genotypes (A) grew significantly slower than the other parental genotype (B) and the hybrid genotype (A×B) at both temperatures. This remained the case at both temperatures for nearly 3000 generations of evolution. After 3000 generations, we still find an effect of genotype on growth rate (ANOVA: F (2,38) = 14.79, P < 0.0001; Table S6), however after investigating the significant interaction effect of genotype by evolution environment (ANOVA: F (2,38) = 6.21, P = 0.0047; Table S6) we found this effect is driven primarily by the 24 ºC evolved populations at this time point. In fact, the significant difference between genotypes is lost after 3000 generations of evolution at 37 ºC (R^2 = 0.0301) but not at 24 ºC (R^2 = 0.472) (Wilcoxon test; Fig. 2.3), indicating that the genotypes converged on a similar growth rate more quickly at the higher temperature. By 4000 generations there is still a significant, but smaller effect of genotype on growth rate (ANOVA: F (2,38) = 3.44, P = 0.0425; Table S7) however Wilcoxon tests detect no significant differences between genotypes at either temperature (Fig. 2.3).

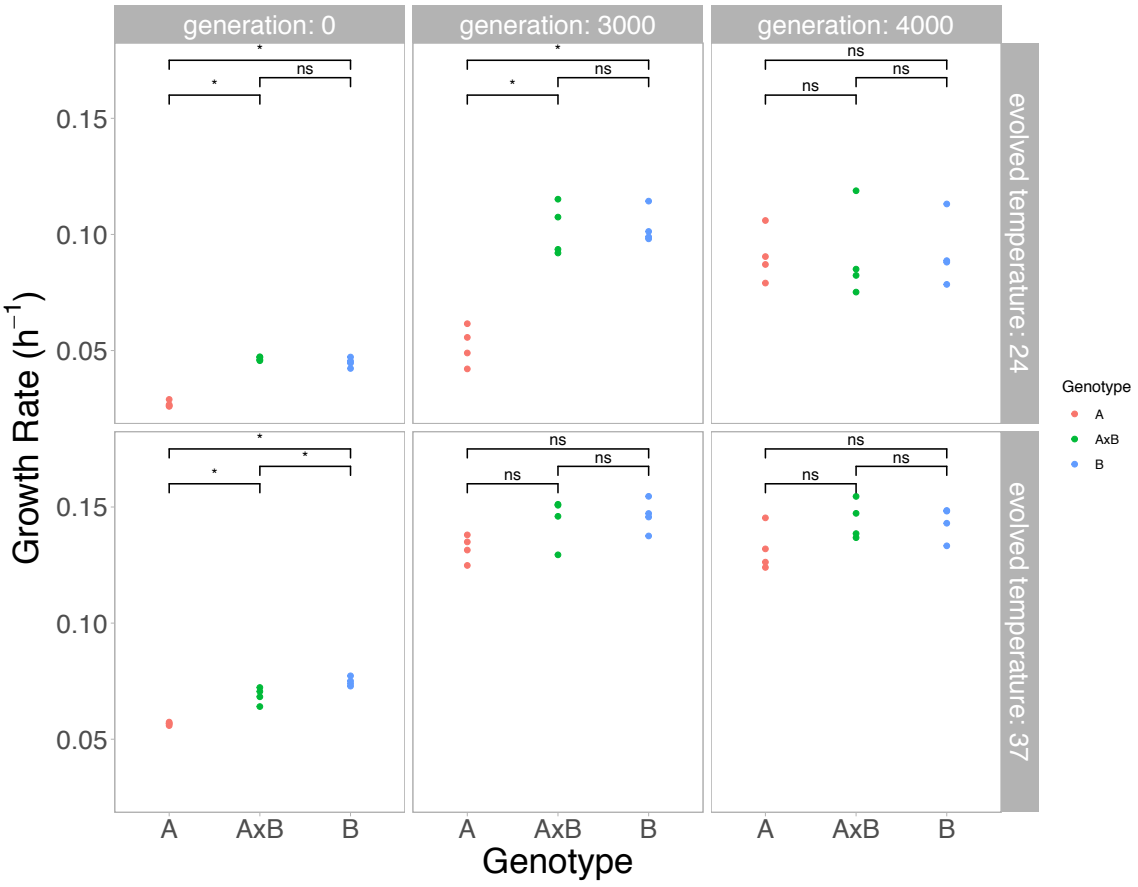

Figure 2.3. Genotypes converge on similar growth rates faster at the higher temperature. Differences in growth rates in the home environment (i.e. assay temperature the same as the evolution temperature) among genotypes are shown at three time points (0, 3000, and 4000 generations) at each temperature. Each point shows the mean growth rate of one replicate population. A Wilcoxon test was used to determine significant differences between genotypes ("*" indicates $p < 0.05$, "ns" indicates no significant difference).

Evolution at a higher temperature results in less variation among replicate populations

The variation in growth rate among replicate populations appeared greater in populations evolved at 24 ºC compared to those evolved at 37 ºC. To test whether apparent differences between replicate populations evolved from a single ancestor were significant we performed a nested ANOVA on mean growth rate per plate at

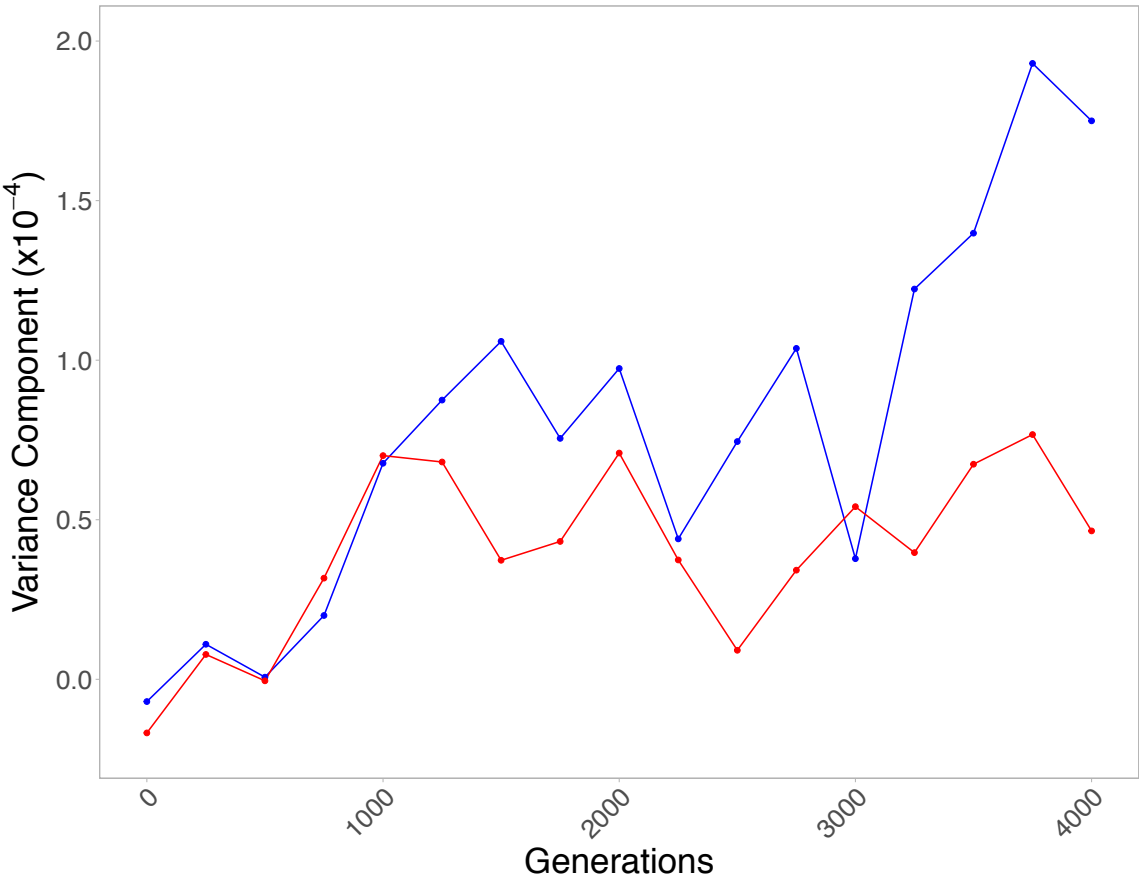

Figure 2.4. Variance in growth rate due to divergence among replicate populations. The variance components attributable to replicate population for populations evolved and assayed at 24 ºC (blue) or 37 ºC (red) over 4000 generations of evolution. Variance components were estimated from an ANOVA with replicate population nested within genotype (*Growth rate ~ genotype, replicate population[genotype]&Random*) for each 250-generation bin and evolution temperature.

4000 generations. We found a significant effect of replicate population nested within genotype ($F_{21,826} = 13.95$, $P < 0.0001$; Table S8) indicating significant divergence between populations evolved from a single ancestor. Similar results were obtained for other time points. In fact, even as soon as generation 125 there is an effect of population nested within genotype ($F_{21,283} = 2.65$, $P = 0.0002$; Table S9) indicating that populations began to evolve measurable differences in growth rate early in their evolution. To further analyze this result and to assess differences in the variance produced at either evolution temperature, we performed Levene's test every

250 generations and compared the variance component attributable to replicate population (nested within genotype) at either evolution temperature (Fig. 2.4). We also compared the variance component attributable to population regardless of genotype (i.e., unnested) for either temperature (Fig. 2.5) to see how the decrease in variation between genotypes (Fig. 2.3) interacts with the variation produced among replicate populations of a given genotype (Fig. 2.4) and effects the overall variation between all populations.

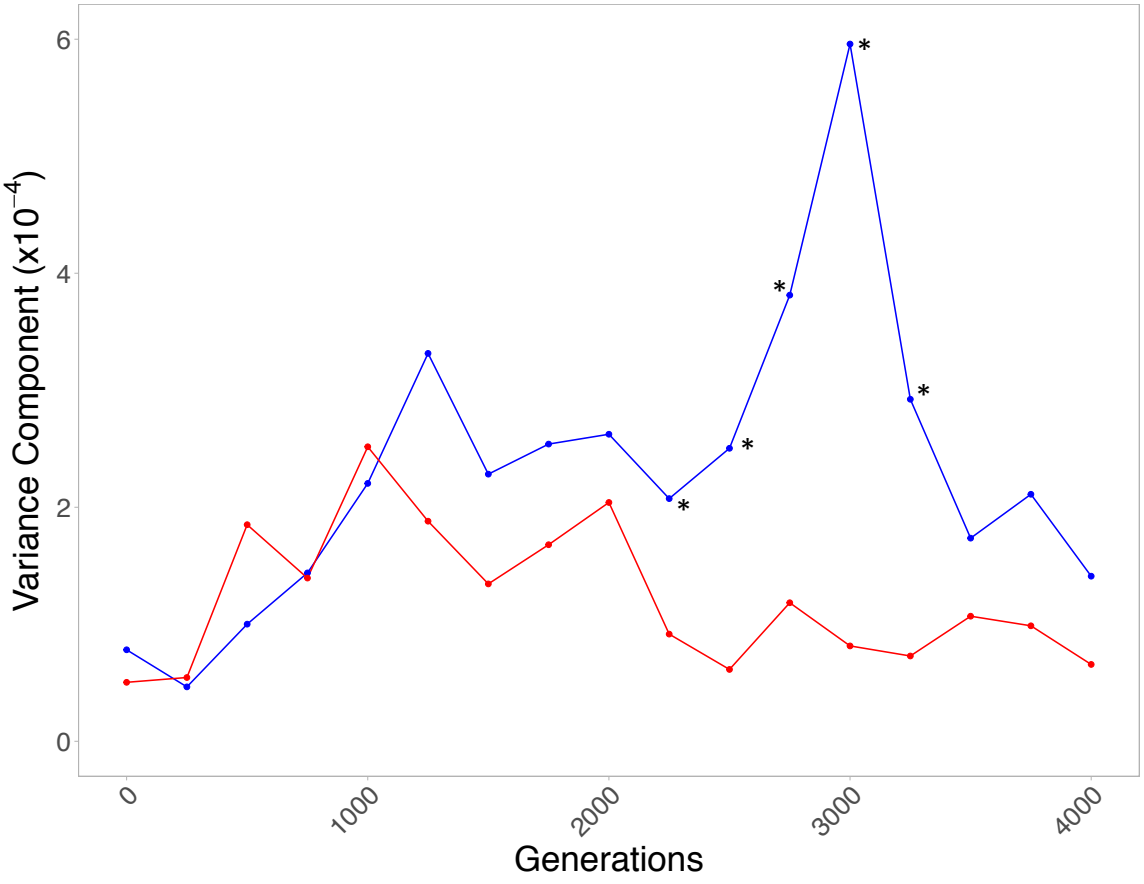

Figure 2.5. Variance in growth rate among all populations is lower for the hotter populations. The variance components attributable to population for populations evolved and assayed at 24 ºC (blue) or 37 ºC (red) over 4000 generations of evolution. Variance components were estimated from an ANOVA without population nested within genotype (*Growth rate ~ population&Random*) for each 250-generation bin and evolution temperature. Asterisks indicate significant results of Levene's test.

The small sample size within a genotype (n=4) meant Levene's test struggled to detect significant differences in the variance between temperatures at each individual time point, but we consistently see a larger variance component attributable to replicate population nested within genotype among populations evolved and assayed at 24 ºC particularly after 1000 generations (Fig. 2.4). This is true regardless of assay temperature, indicating that evolution temperature is driving this effect, and demonstrating greater repeatability in the growth rate trajectories of populations evolved at 37 ºC.

When we combine growth rate data from all genotypes Levene's tests indicate there is a significant difference in the variance among populations at either temperature from generation 2250 to generation 3250 (Fig. 2.5). We also find consistently lower variance components attributable to population among 37 ºC-evolved populations than those evolved at 24 ºC (Fig. 2.5). This is due to the joint effect of less divergence between replicate populations of the same genotype (Fig. 2.4) and more convergence among different genotypes for populations evolved at 37 ºC relative to those evolved at 24 ºC (Fig. 2.3). At both temperatures the variance component attributable to population peaks at an intermediate generation, although the peak is higher and later for populations evolved at 24 ºC, as variation accumulates among replicate populations but before genotypes have had sufficient time to converge (Fig. 2.5).

In spite of the greater variation among replicate populations of the same genotype evolved at 24 ºC (Fig. 2.4) we still detect greater differences among genotypes when evolution takes place at 24 ºC (Fig. 2.3). This indicates that the observed differences among genotypes at 24 ºC vs. 37 ºC (described in the section above) are not just due to higher variability among replicate populations at the lower temperatures, but also to longer lasting differences between genotypes.

Asymmetry of the correlated responses

By generation 4000, all populations increased in growth rate at both the temperature in which they evolved and the alternate temperature, indicating no evidence of trade-offs at this time point. However, we find a marginally significant interaction between evolution temperature and assay temperature (ANOVA: F (1,38) =3.17, P = 0.0829; Table S7) at generation 4000. This suggests that some of the adaptation that has taken place over the course of the experiment is temperature-specific despite an overall correlation between growth rates of evolved populations at either temperature (r = 0.597). This correlation is even greater when the ancestors are included in the analysis (r = 0.858; Fig. 2.6).

To assess which temperatures were driving the interaction between evolution temperature and assay temperature, we compared growth rates from each assay

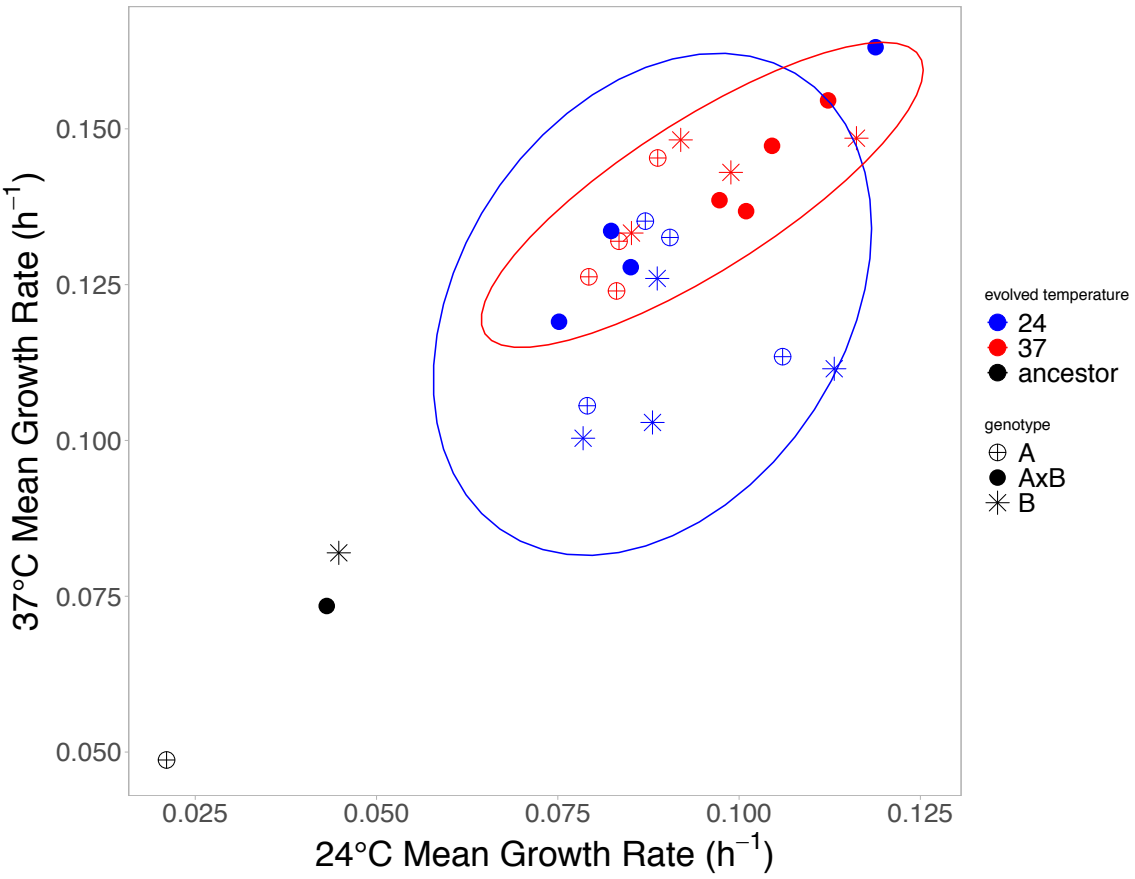

Figure 2.6. Correlation between growth rates in alternative environments. Growth rate of populations after 4000 generations of evolution, measured at 37 ºC (y-axis) or 24 ºC (x-axis). Genotypes are indicated by the symbols and the evolution environment is indicated by red (37 ºC) or blue (24 ºC) with the ancestors shown in black. A trade-off exists if an evolved population has lower fitness than its ancestor at the alternate temperature from which it evolved. No trade-offs are observed here. The 95% confidence ellipse is shown for populations evolved at 37 ºC (red) and for populations evolved at 24 ºC (blue).

temperature. We found a significant effect of evolution temperature when assays were performed at 37 ºC ($R^2 = 0.285$) but, remarkably, not at 24 ºC ($R^2 = 0.0265$; Tukey-Kramer: $p < 0.05$). This means that even after 4000 generations of evolution, the temperature at which evolution occurred makes no difference when growth rate is assayed at 24 ºC. This indicates there is a greater correlated response when evolution occurs at 37 ºC. In other words, evolution at the hotter temperature increased growth at the colder temperature more than evolution at the colder temperature increased growth at the hotter temperature (Fig. 2.6).

Discussion

We examined the evolutionary trajectories of populations of different genotypes of *T. thermophila* under differing temperature regimes. Our experimental design allowed us to test how evolution temperature affects repeatability, as well as how it impacts historical differences as evolution progressed at each temperature. We found that the hotter temperature resulted in greater repeatability of evolution and faster convergence between divergent genotypes.

After 4000 generations, we found that populations evolved at 37 ºC significantly outperformed those evolved at 24 ºC (Fig. 2.1). This outcome aligns with previous findings that "hotter is better" (Knies et al. 2009; Angilletta et al. 2010). This theory states that hot-adapted genotypes will have higher maximum growth rates than cold-adapted genotypes because they have evolved greater robustness in response to the chemical and metabolic reactions happening more quickly at hotter temperatures and because the rate-depressing effects of low temperature cannot be overcome by adaptation or plasticity.

Temperature affects the convergence of different genotypes

Over the course of evolution, different starting genotypes and phenotypes could converge, evolve in parallel, or diverge even further. Through epistatic interactions, genotype can constrain the future evolution of a population by biasing the set of available beneficial mutations that are likely to be selected (Draghi and Plotkin 2013).

Similar genotypes are expected to fix a similar set of mutations while more divergent genotypes are expected to fix a less similar set of mutations leading to further divergence between the genotypes (Blount et al. 2018; Starr et al. 2018). At the same time natural selection could overcome both random drift and epistatic interactions to produce convergence between divergent genotypes.

Previous experiments have found that the rate of adaptation is inversely proportional to fitness and that initially different populations often end up at the same fitness optima (Jerison et al. 2017; Wünsche et al. 2017). At the same time studies have also found that particular alleles can impede this fitness recovery and constrain the future of evolution (Woods et al. 2011; Jerison et al. 2017). However, these experiments were limited to less than 1000 generations of evolution and it is unclear whether continued evolution would eventually allow these populations to reach the same fitness optima as their relatives. For more distantly related populations, we might expect this process to take longer if it even occurs at all.

In our experiment, the maintenance of historical differences between divergent genotypes of the same species over many generations of evolution at both temperatures suggests that genetic differences in the initially slowest growing genotype are impeding future adaptation in a manner that is not easily overcome. Despite the overall increase in growth rate being greatest for the initially less fit genotype as expected, we observe slower rates of adaptation for this genotype than we would expect if all genotypes followed the same pattern of diminishing returns

epistasis. We also find that temperature affects this pattern and the rate of convergence. Differences in growth rate between genotypes were maintained for over 3000 generations at 24 ºC while convergence among the genotypes was more rapid at 37 ºC. Why a higher temperature would be more conducive to convergence is unclear but could be related to other effects of temperature observed in our experiment. For example, higher selection coefficients and/or more targets of selection at 37 ºC may contribute to the slower growing genotype catching up more quickly at this temperature, to the greater repeatability, and to the asymmetry of the correlated responses.

The ability of populations to escape constraints on evolutionary change can be vital to long-term survival (Chao and Weinreich 2005; Weinreich et al. 2005). In this experiment, we show the gradual loss of growth rate differences between genotypes even while differences evolve among replicate populations of the same genotype at both temperatures. This suggests differing levels of divergence, wherein it is promoted locally, as replicate populations diverge, but diminished globally, as the mean growth rates of divergent genotypes converge in the same environment. Whether this would also be true for more distantly related populations remains unclear but the notion that there is an intermediate level of relatedness at which point the future phenotypic divergence between two populations is minimized is intriguing.

Temperature affects repeatability among populations

Previous studies have found differences in the repeatability of evolutionary trajectories under different environmental conditions (e.g., Gresham et al. 2008; Bailey et al. 2015). In these experiments, replicate populations were more likely to diverge in some environments but experience repeatable evolutionary trajectories in others. Likewise, we found that replicate populations of all genotypes diverged more at 24 ºC and were more repeatable at 37 ºC.

The greater variation among populations evolved at 24 ºC suggests that these evolutionary trajectories are more dependent on chance events than the populations evolved at 37 ºC. This result may reflect differences in the environment that affect the degree of epistasis or "ruggedness" of the fitness landscape and/or rate of mutation and distribution of their effects.

Differences in the "ruggedness" of the fitness landscape, caused by epistatic interactions, at each temperature could explain our observation of increased repeatability at 37 ºC. While theory predicts that a rugged fitness landscape can increase the repeatability of evolution at the level of the mutational pathways followed (De Visser and Krug 2014) the opposite is true at the fitness level (Bank et al. 2016). So, the greater repeatability at 37 ºC could result from a more uniform fitness landscape at this temperature.

Greater repeatability could also result from a difference in the distribution of beneficial mutations available in each environment (Lenski et al. 1991). At 24 ºC, the lower repeatability suggests there may be rare highly beneficial mutations that increase growth rate in some but not all populations, while at 37 ºC there may be fewer of these types of mutations resulting in growth increasing more uniformly across replicate populations. If this were the case, we would eventually expect to see a reduction in the variation among replicate populations evolved at 24 ºC. Continued experimental evolution of our populations may eventually lead to this result, but if epistatic interactions are important, as they appear to be (Kuzmin et al. 2018), they may constrain future evolution making eventual convergence even more unlikely.

The strength of selection may also differ in these environments. Theoretical results suggest that stronger selection results in increased repeatability (Orr 2005). This theory is corroborated by a meta-analysis showing a strong positive relationship between population size, with larger populations experiencing greater selection, and greater repeatability (Bailey et al. 2017). Our populations are approximately the same size at either temperature meaning our observations are not simply a reflection of differences in the sizes of the populations at either temperature. However, 37 ºC is near the upper limit of the thermal tolerance for this species (Hallberg et al. 1985), which may pose a greater selective pressure thereby causing the observed reduction in variation among populations evolved at this temperature.

Temperature affects correlated responses

Experiments using *E. coli* have found substantial evidence for temperature associated trade-offs (Bennett et al. 1992; Bennett and Lenski 1993, 2007; Mongold et al. 1996; Woods et al. 2006). In *T. thermophila,* we find no evidence for trade-offs in any of our populations after 4000 generations. However, we do find an asymmetric correlated response, whereby evolution at 37 ºC increases growth rate at 24 ºC more than evolution at 24 ºC increases growth rate at 37 ºC, which is similar to what is observed in *E. coli.* Evolution at a hotter temperature increases growth rate a colder temperature for both species while evolution at a colder temperature increases growth rate at a hotter temperature less for *T. thermophila* and often decreases it for *E. coli* (Bennett et al. 1992; Bennett and Lenski 1993; Mongold et al. 1996). One likely explanation for the difference between *T. thermophila* and *E. coli* is that the *E. coli* experiments started from an ancestor that had already evolved under laboratory conditions for 2000 generations and was therefore pre-adapted to the general culture conditions, as opposed to our *T. thermophila* lines, which were derived from wild collected strains grown in lab only ~500 generations before cryopreservation. Thus, it seems likely that a greater proportion of the adaptation that occurred in the *T. thermophila* populations, compared to the *E. coli* populations, involved adaptation to the general culture conditions as opposed to the specific temperature.

As evolution occurs in one environment, fitness may change in other environments either as a direct pleiotropic response to selection in the evolution environment or due to the accumulation of mutations that are neutral in the evolution environment

but have fitness consequences in the other environment (Cooper and Lenski 2000). The asymmetry we observe in the correlated responses could be due to asymmetry in the pleiotropic responses, whereby a 37 ºC beneficial mutation increases growth rate more at 24 ºC than a 24 ºC beneficial mutation does at 37 ºC. Alternatively the asymmetry in the correlated responses could arise from an asymmetry in the effect of neutral and nearly neutral mutations at the alternate temperature. In other words, the neutral and nearly neutral mutations that are able to accumulate at 37 ºC are also mostly neutral at 24 ºC while the neutral and nearly neutral mutations that are able to accumulate at 24 ºC tend, on average, to be slightly deleterious at 37 ºC. These two possibilities are not mutually exclusive.

One possible mechanistic explanation for the observed asymmetry could be more transcript diversity, and thus more targets of selection, in hotter conditions resulting in most genes that are transcribed and selected at 24 ºC also being transcribed and selected at 37 ºC but not vice versa. This would be consistent with the lack of antagonistic pleiotropy across temperatures among the most positively selected mutations found in lab-evolved *E. coli* (Deatherage et al. 2017) and is supported by data showing that more genes are up-regulated at hotter temperatures (Tai et al. 2007; Mittal et al. 2009). Additionally, the 37 ºC evolved populations experience a greater density range, and thus a more heterogenous environment, than those evolved at 24 ºC, which could also contribute to greater transcript diversity and the asymmetry in the correlated response that we observe. This idea is supported by a meta-analysis of trade-off experiments, which found that populations evolved in

homogeneous environments exhibited more trade-offs than populations evolved in temporally heterogeneous environments (Bono et al. 2017). The 37 ºC populations also experience an additional possible source of heterogeneity because the 37 ºC tubes are not pre-heated so the cells experience the 24 ºC temperatures for a very brief period each day. It is conceivable that this very brief period of cold is sufficient to explain the greater correlated response in the 37 ºC evolved populations. However, we consider this unlikely as this cold exposure is taking place during lag phase, not when cells are dividing, and is therefore unlikely to impact selection on the growth rate.

The asymmetric correlated response we observe may also be related to the other effects of evolution temperature that we observed. For example, the conditions responsible for greater convergence and repeatability when evolution occurs at 37 ºC may also act to optimize and constrain growth rate at the lower temperature. Thus, our results are consistent with there being more targets of selection at 37 ºC, which would lead to faster adaptation, greater repeatability, and asymmetric correlated responses. It is also possible that all of these results are a reflection of the “hotter is better” theory (Knies et al. 2009; Angilletta et al. 2010). However, this theory does not directly explain the observed correlated responses of evolution in hotter conditions indicating that different aspects of the 37 ºC environment may be responsible for the greater convergence, the greater repeatability, and the larger correlated response. In the future, more high-throughput methods with greater control of the evolution conditions will allow for the identification of the precise environmental conditions

responsible for the difference that we observed in evolution at different temperatures.

Another possible interpretation of our results is that populations evolving at 24 ºC adapt by increasing different components of fitness than those evolving at 37 ºC. We measured growth rate, which is a major component of fitness, and well correlated with competitive ability in our experiments, but fitness can also increase in more complex ways than simply increasing maximum growth rate (Li et al. 2018). For example, decreasing lag time or increasing carrying capacity could increase fitness without effecting growth rate. Additionally fitness gains can be accrued and realized in different portions of the growth-cycle (Li et al. 2018), which could contribute to the asymmetry of the correlated responses that we observe if the amount of time spent in different phases of the growth cycle differs substantially between temperatures. A final caveat is that all of the adaptation that we observed occurred in the somatic nucleus, which is discarded following sexual reproduction. While there is evidence of some epigenetic inheritance between parental and progeny somatic genomes (Beisson and Sonneborn 1965; Chalker and Yao 1996; Pilling et al. 2017), it is unknown whether any of the adaptation that occurred in our experimental populations would be inherited by newly produced sexual progeny. However, this may be a moot point in this experiment because all of the evolved populations lost the ability to undergo sexual conjugation, at least under laboratory conditions.

Chapter III

A single sexual progeny has increased evolvability in the ciliate *Tetrahymena thermophila*

Introduction

Fisher's fundamental theorem states that the rate at which a population increases in fitness is equal to its genetic variance in fitness at that time (Fisher 1958). Ultimately, all adaptations result from the initial appearance and eventual fixation of novel genetic variants. Genetic variants arise normally in populations through mutation, gene flow, and sex. In fact, it is hypothesized that sex is maintained, despite its cost, to generate genetic variance for fitness and increase evolvability (Colegrave 2002). *Tetrahymena thermophila* is a facultatively sexual, free-living, single-celled eukaryote with an unusual genome architecture that allows for the generation of additional genetic variation following sex that should increase its evolvability. This increased evolvability may also provide a novel explanation for the maintenance of this unusual genome architecture.

Like other ciliates, *Tetrahymena* contains two types of nuclei: a silent germline micronucleus (MIC) and a transcriptionally active somatic macronucleus (MAC) (Merriam and Bruns 1988). The MAC gets destroyed after sex and a new one gets created from a mitotic product of the new zygotic nucleus. In the model ciliate *T. thermophila,* the MAC contains ~45 copies of every chromosome (~225) and divides by amitosis (Fig. 3.1;Orias and Flacks 1975; Eisen et al. 2006). During amitosis the

chromosomes do not line up and segregate as they do during mitotic metaphase and anaphase. Instead the content of the genome is divided apparently randomly between the daughter cells (Karrer 2012). Over time this will result in one or the other parental allele being lost entirely from the MAC until the whole genome, except for *de novo* mutations, is homozygous for one or the other parental allele (Fig. 3.1;Sonneborn 1974). This process is known as phenotypic or allelic assortment and occurs independently for all ~225 chromosomes. Amitotic division is thus predicted to result in large amounts of combinatorial genetic variation in the vegetative growth of a single sexual progeny. This increase in genetic variation should increase the rate at which an amitotically dividing population adapts (Doerder 2014, Zhang et al. submitted).

Models have shown the novel genetic architecture of ciliates results in population genetics that differ from canonical population models (Morgens et al. 2014). Other models have shown that amitosis in *Tetrahymena* allows asexual lineages to slow Mueller's ratchet and adapt at a rate similar to sexual lineages (Zhang et al., submitted). Additionally, several studies have claimed the genetic architecture of ciliates drives rapid gene and protein evolution (Zufall et al. 2006; Gao et al. 2014).

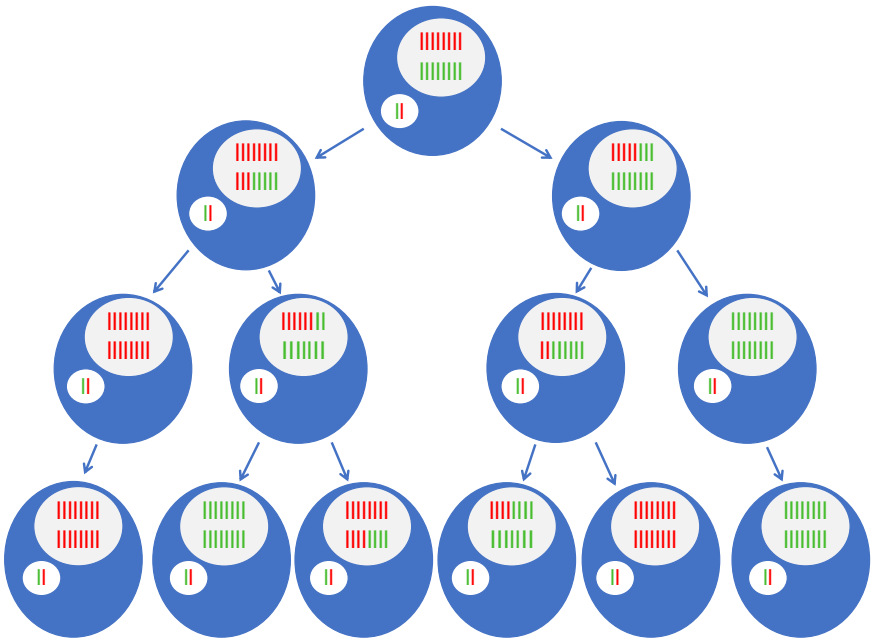

Figure 3.1. Amitosis and phenotypic assortment at a single locus. Figure show the gradual loss of heterozygosity following sex. The small white oval is the diploid micronucleus containing the red allele inherited from one parent and the green allele inherited from the other. The large white oval is the polyploid (n=45, only 16 are shown) macronucleus (MIC). Following sex the macronucleus (MAC) develops from the new zygotic MIC and contains approximately half of the alleles from one parent and half from the other (shown in red and green). As the cell divides amitotically each daughter cell (indicated by the arrows) inherits a random mixture of parental alleles. This process is accelerated in the figure above, while in reality it is likely to take ~200 generations for 99% of loci to become homozygous. Amitosis results in phenotypic assortment and is likely to increase the genetic variation during the vegetative growth period following sex. The DNA content of an asexually dividing organism normally changes only through various mutational processes, but in *T. thermophila* a single heterozygous MAC genotype can give rise to a huge number of alternative genotypes. The high chromosome number in addition to recombination between homologous chromosomes creates minimal physical linkage between loci allowing for differential assortment of most alleles. This results in a large number of possible combinations of parental alleles being produced in the vegetative growth from a single mating. With selection acting on this variation, alleles and combinations of alleles will come to dominate in environments where they are advantageous, increasing the fitness of the population.

My central aim is to experimentally test the hypothesis that *T. thermophila* genome architecture and amitosis affects the dynamics of adaptation and the consequences of sex. Specifically, I am testing the hypothesis that populations founded from a single sexual progeny are more evolvable than populations founded from either unmated parent. To test whether amitosis will indeed increase the rate of adaptation following sex, I compare the slopes of the fitness trajectories of progeny and parental

populations. The parental populations have already undergone phenotypic assortment and are thus largely homozygous in the MAC. This means that amitosis will have little to no effect on the asexual progeny. In contrast, the sexually produced progeny should be highly heterozygous, meaning that amitosis will produce progeny with differing combinations of alleles. If amitosis increases evolvability following sex, then the rate of fitness increase in a population founded by a single new sexual progeny will be greater than that founded by an individual that has not had sex in many generations.

Methods

Summary

Three independent experiments were performed to compare the rate of adaptation in populations derived from a sexual progeny to populations derived from individuals that had been dividing asexually only. Each experiment started with different parental genotypes of *T. thermophila*, which were crossed to produce a sexual progeny. A single cell was used to found the progeny populations. Populations were allowed to evolve for 400-1000 generations, during which population growth rates were measured.

Strains and initial cross

For experiment 1 natural isolates of *T. thermophila* designated 19617-1 (Tetrahymena Stock Center ID SD03089; Cox1 GB: KY218380) and 19625-2 (Cox1 GB: KY218383) (Doerder 2019) were thawed from frozen stocks, inoculated into 5.5

mL of the nutrient rich media SSP (Gorovsky et al. 1975) in a 50 mL conical tube, and incubated at 30 ºC with mixing for two days. These cultures were maintained as the parental lines. Eight populations were established for each genotype in 10 mL of SSP. Four populations were maintained at 24 ºC and four at 37 ºC. Populations were designated by genotype (19617-1 or 19625-2 herein referred to as A and B, respectively) – replicate (1-4) – and evolution temperature (24 ºC or 37 ºC), e.g., A-1-37.

To generate the hybrid genotype from these strains, a conical tube of each parental genotype was centrifuged and the supernatant was poured off before the cells were re-suspended in 10 μM Tris buffer (Bruns and Brussard 1974). After mixing at 30 ºC in Tris for two days to starve the cells and induce sexual competence, 1 mL of each starved parental population and an additional 1 ml of 10 μM Tris buffer were added to one well in a six-well plate and placed back in the 30 ºC incubator. The next morning (~12 hours later) the plate was checked for pairs and put back in the incubator for an additional 4 hours to allow progression of conjugation. Individual mating pairs were isolated under a microscope using a 2 μL- micropipette and placed in 180 μL of SSP in one well of a 96-well plate. The plate was then incubated for 48 hours after which time a single cell was isolated from each well and re-cultured into 180 μL of fresh SSP in a new well. After another 48 hours at 30 ºC four individual cells were isolated from one of the wells, into new wells with SSP, one for each of the replicate populations, and incubated at 30 ºC for 48 hours. Each of the four 180 μL cultures was then split in two with each half being added to a separate 50 mL conical tube

containing 10 mL of SSP, one designated for evolution at 37 ºC and the other at 24 ºC. These eight cultures are the starting hybrid populations and are designated as A×B (19625×19617) – replicate (1-4) – evolution temperature.

This provided us with a total of 24 populations consisting of three genotypes, two parental and one hybrid, half of which were evolved at 24 ºC and half at 37 ºC with four replicate populations of each genotype per treatment.

Progeny were generated as described above for experiment 2 using natural isolates 20453-1 (C; *Tetrahymena* Stock Center ID SD01561; collected in New Hampshire; Cox1 GB: KY218424) and 20438-1 (D; ID SD01559; collected in New Hampshire; Cox1 GB: KY218417) and for experiment 3 using isolates 20395-1 (E; ID SD01557; collected in New Hampshire; Cox1 GB: KY218412) and 20488-4 (F; ID SD01566; collected in Vermont: Cox1 GB: KY218435). In both experiment 2 and 3, 16 replicate populations of each parent and the single progeny were maintained (48 populations in total).

Prior to the start of the experiment parental strains had been kept in lab in cultures containing only a single mating type for at least 200 generations so we know they have not had sex in at least that long and should therefore be highly homozygous. We confirmed that progeny had indeed had sex by performing maturity tests. After sex *Tetrahymena* experience a period of immaturity when they will not pair or have

sex again (Doerder et al. 1995). Immaturity tests confirmed that our sexual progeny would not pair indicating it has indeed just had sex.

Transfer regime

In experiment 1 approximately 20,000 cells (~90 μL) from each 37 ºC culture and 60,000 (~1 mL) from each 24 ºC culture were transferred to 10 mL of fresh SSP daily. Transfer volumes were adjusted as needed to maintain the same starting culture density at each transfer. On average, the 37 ºC evolved populations achieved ~6.8 generations per day and the 24 ºC populations achieved ~3.5 generations per day. This means that 37 ºC evolved populations experienced a wider range of densities (~2,500 cells/mL – ~275,000 cells/mL) than 24 ºC evolved populations (~6,000 cells/ mL – ~60,000 cells/mL), starting with a lower density and ending at a higher density. We estimate the effective population size to be approximately 100,000 cells for each evolved environment by calculating the harmonic mean of the population size at each discrete generation (Karlin 1968).

In experiments 2 and 3 ~1200 cells (2.625 μL) from each culture were transferred to 180 μL of fresh SSP daily and incubated at 30 ºC on the microplate reader. This resulted in a starting density of ~6700 cells/mL, a final density of ~425,000 cells*/mL,* and 6 generations each day. These populations were evolved for at least 370 generations on a 96-well plate with an estimated effective population size of ~3000.

Growth curves and analysis

For experiment 1 growth rate was measured by inoculating ~500 – 1000 cells into one well of a 96-well plate and measuring the optical density (OD) at 650 nm in a micro-plate reader every 5 minutes over the course of 24 – 48 hours for 37 ºC assays and 48 – 72 hours for 24 ºC assays (see Chapter II for validation of use of OD_{650} as a proxy for cell density). The maximum growth rate was then estimated for each well by fitting a linear regression to the steepest part of the growth curve, estimating the maximum doublings per hour (h^{-1}) (Wang et al. 2012; Long et al. 2013). 3 – 4 replicates of all populations were measured on a plate at each time point and the mean growth rate per plate was used in our analysis. For experiments 2 and 3, populations were evolved on the microplate reader and growth rates were measured for each population as evolution progressed as described above. Maximum optical density and onset time to 0.12 OD were measured in addition to growth rate in experiment 2 and 3.

Data analysis

For each of the three experiments described above the results were analyzed separately by comparing the fitted slope of the growth rate trajectory of the progeny populations to that of the parents. The slope of the growth rate trajectory (or the evolvability) of each genotype was estimated from the data using a linear model (*growth rate ~ genotype, generations, genotype*generations)* to estimate growth rate which included genotype, generations, and the genotype*generations term (which corresponds to the slope of the growth rate trajectory or the evolvability). This

approach provided us with a standard error of our estimate allowing us to assess whether the slopes or evolvabilities of different genotypes are significantly different from each other. For experiment 1, which included more generations and larger population sizes than experiments 2 and 3, the natural log of generation was used in our analysis to linearize the data. This allowed us to remove the quadratic term from our model without reducing the fit. A regression analysis found no correlation between the residuals and generations after transformation. The absolute increase in growth rate (i.e., evolved - ancestral growth rate) was calculated for each population by binning the final ~100 generations of each experiment. A pairwise Student's t-test was used to test for significant differences in the total increase in growth rate between each genotype. For experiments 2 and 3 the same analysis was performed on maximum OD and onset time data collected over the same period.

Expectations

Computational predictions suggest ~99% of neutral loci in progeny genotypes should be fixed within an individual for one parental allele after ~439 generations (personal com. Ricardo Azevedo). This happens independently for each of the ~225 chromosomes (Eisen et al. 2006) resulting in an enormous amount of combinatorial variation in the ~439 generations following sex. Thus, we expect that populations founded by a sexual progeny will increase in fitness faster than populations founded by either parent. The genetic variation in new sexual progeny populations should be greatest when phenotypic assortment is nearing completion but before less fit variants are completely lost from the population. After phenotypic assortment is

complete the time to fixation for an individual with a 5% fitness advantage is ~921 generations for the larger populations (N_e = 100,000) and ~641 generations for the smaller populations (N_e = 3,000). These estimates are based on the formula $t \sim (4\ln(2N))/s$ (Kimura and Ohta 1969), do not consider clonal interference, treat individuals as haploid, and are conservative because they assume an initial frequency of 1/N.

Results

As predicted, in all three experiments, the populations founded from a single heterozygous sexual progeny cell adapted more quickly than the homozygous parental populations (Fig. 3.2-3.9). In experiment 1, all populations showed greater increases in growth rate over the course of 1000 generations than in experiments 2 and 3. The larger growth rate increases in experiment 1 are likely due to the larger population size, the additional generations of evolution, and the novelty of the 24 °C and 37 °C environment when compared to the 30 °C environment. The same qualitative result was seen at both temperatures demonstrating that the increased evolvability of the progeny populations is not environment dependent. We found that the progeny populations (AxB) increase in growth rate significantly faster than either parent (A and B) at both temperatures (Fig. 3.2; estimate of slopes shown in table 3.1).

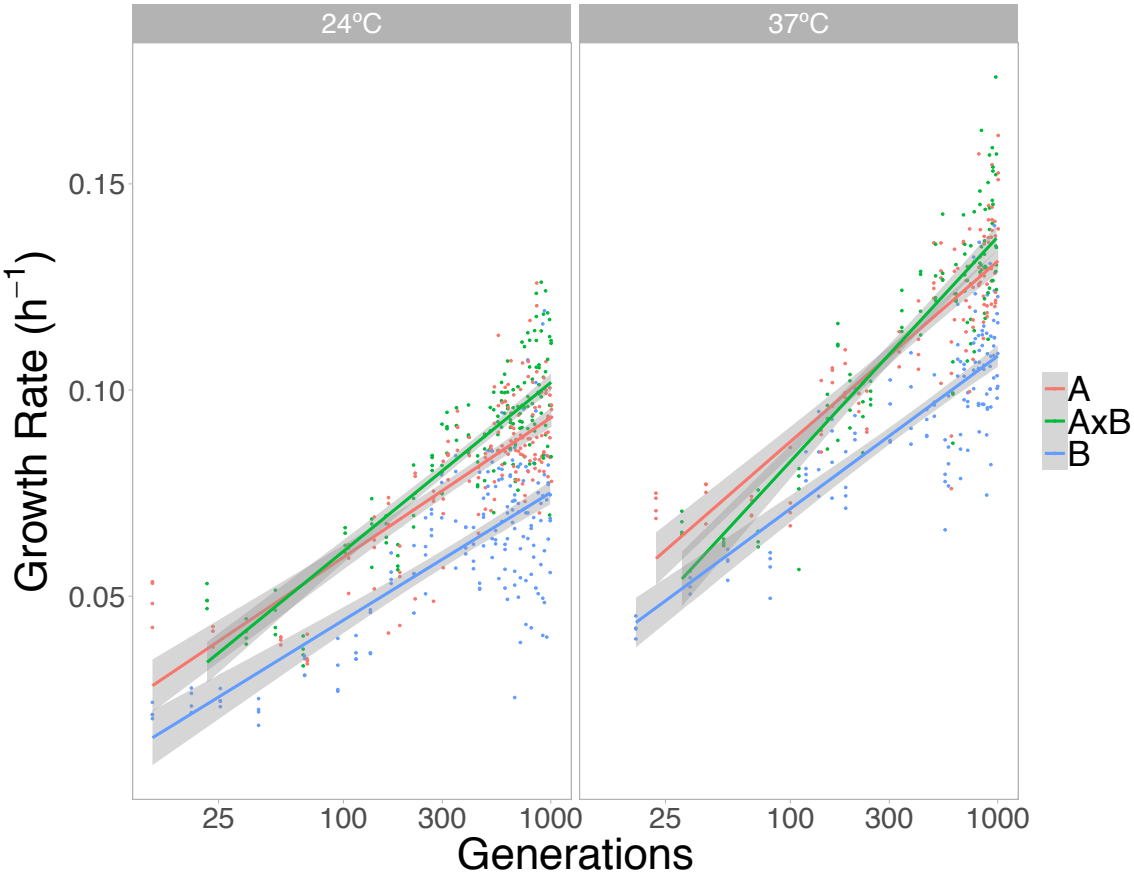

Figure 3.2. Experiment 1. Growth rate trajectories of the parental (red and blue) and progeny (green) genotypes over 1000 generations of evolution at 24 ºC (left panel) and 37 ºC (right panel). Each point is the mean growth rate per timepoint of one of the replicate populations. A linear regression with a 95% confidence interval (shown in gray) is shown for each genotype at either temperature. At both temperatures the slope of the regression of the progeny populations is significantly steeper than either parent.

We also compared the absolute increase in growth rate between the progeny and either parent at the final time point. While the progeny populations increased more in growth rate on average at either temperature the small number of replicate populations (n=4) did not provide us with sufficient power to say whether this difference is significant (pairwise student t-tests shown in Fig. 3.7).

Table 3.1. Estimates of evolvability.

	Sexual progeny x generation	Parent 1 x generation	Parent 2 x generation
Experiment 1 (24 ºC)	**0.0178** SE 0.00076	**0.0147*** SE 0.000744	**0.0134*** SE 0.000699
Experiment 1 (37 ºC)	**0.0237** SE 0.000897	**0.0190*** SE 0.000867	**0.0161*** SE 0.000829
Experiment 2 Growth rate	**2.30×10^{-5}** SE 2.06×10^{-6}	**2.06×10^{-5}** SE 2.06×10^{-6}	**1.01×10^{-5}*** SE 2.06×10^{-6}
Experiment 2 Maximum OD 650	**-9.46×10^{-5}** SE 7.50×10^{-6}	**-1.78×10^{-5}** SE 7.50×10^{-6}	**-7.66×10^{-5}** SE 7.51×10^{-6}
Experiment 2 Onset time	**0.00276** SE 0.000210	**0.00209** SE 0.000210	**0.00198** SE 0.000211
Experiment 3 Growth rate	**4.89×10^{-5}** SE 6.42×10^{-6}	**2.15×10^{-5}*** SE 6.42×10^{-6}	**3.82×10^{-5}*** SE 6.42×10^{-6}
Experiment 3 Maximum OD 650	**6.60×10^{-5}** SE 2.09×10^{-5}	**-0.000124*** SE 2.09×10^{-5}	**-6.00×10^{-5}*** SE 2.09×10^{-5}
Experiment 3 Onset time	**5.95×10^{-5}** SE 0.000611	**0.00318*** SE 0.000611	**0.00316*** SE 0.000613

Table 3.1. Estimates of evolvability. Estimate of the increase in fitness parameters per generation (or ln(generation for experiment 1) are shown in bold followed by its standard error. These estimates correspond to the slopes in figures 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, and 3.8 and are our measures of evolvability. The "*" indicates if the estimate of evolvability for parent 1 or 2 is significantly different than the estimate for the populations founded by a new sexual progeny. A standard least squares model including the effects of genotype, generation (ln(generations was used in experiment 1), and the interaction between them on r-max was used. Parent 1 column shows data for parents A, C, and E.

For both experiment 2 and 3 the progeny populations (CxD and ExF) increased in growth rate faster than either of their respective parents (Fig. 3.3 and 3.4; estimate of slopes shown in table 3.1). In addition, progeny populations had larger increases in growth rate than either parent in the final timepoint of both experiments (pairwise student t-tests shown in Fig. 3.7). In experiment 3, but not experiment 2, populations founded by the sexual progeny also experienced significantly faster increases in the

maximum OD recorded per growth cycle (Fig. 3.4 and Fig. 3.7) and significantly faster decreases in the onset time to 0.12OD (Fig. 3.5 and Fig. 3.8) as evolution progressed. The sexual progeny started out with a higher growth rate and maximum OD in experiment 2, therefore these results may reflect the overriding tendency of diminishing returns epistasis to increase the evolvability of less fit populations.

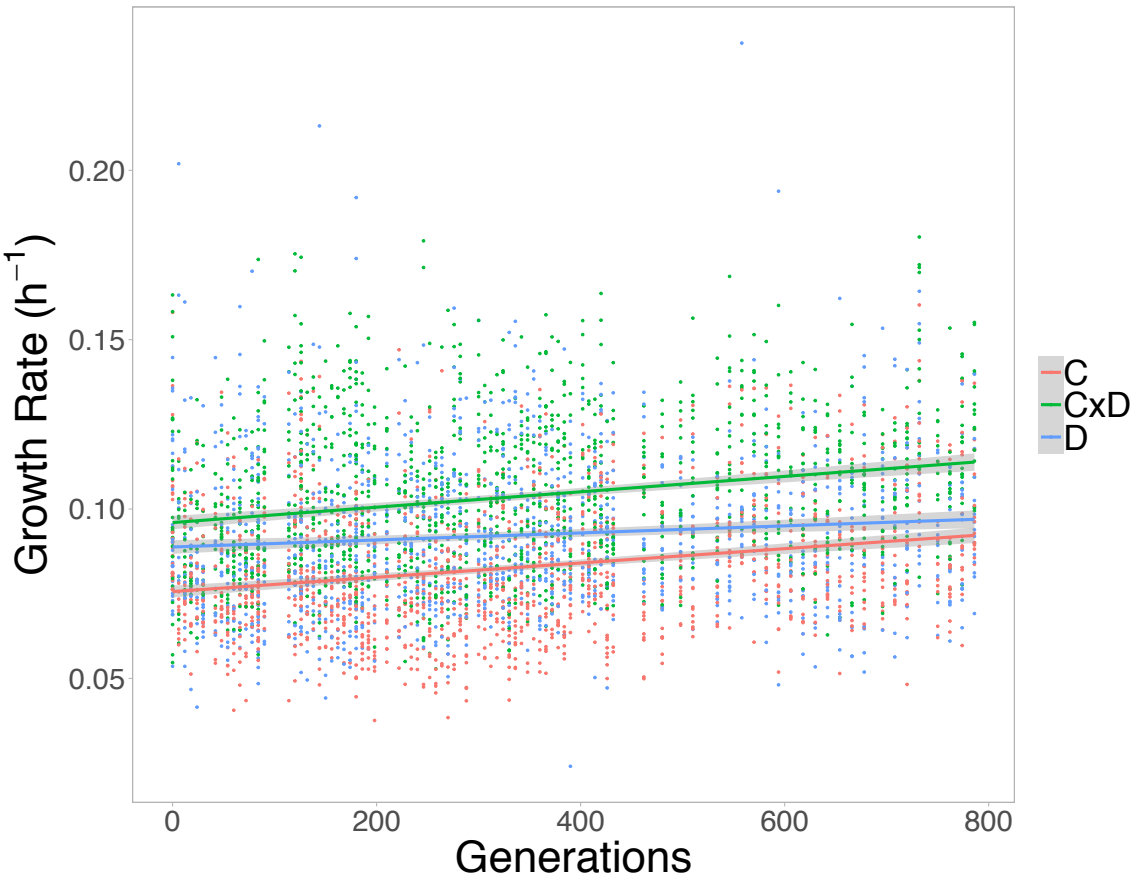

Figure 3.3. Experiment 2. Growth rate trajectories of the parental (red and blue) and progeny (green) genotypes over nearly 800 generations of evolution. Each point is the mean growth rate per timepoint of one of the replicate populations. A linear regression with a 95% confidence interval is shown in gray. The slope of the regression of the progeny populations is significantly steeper than parent D.

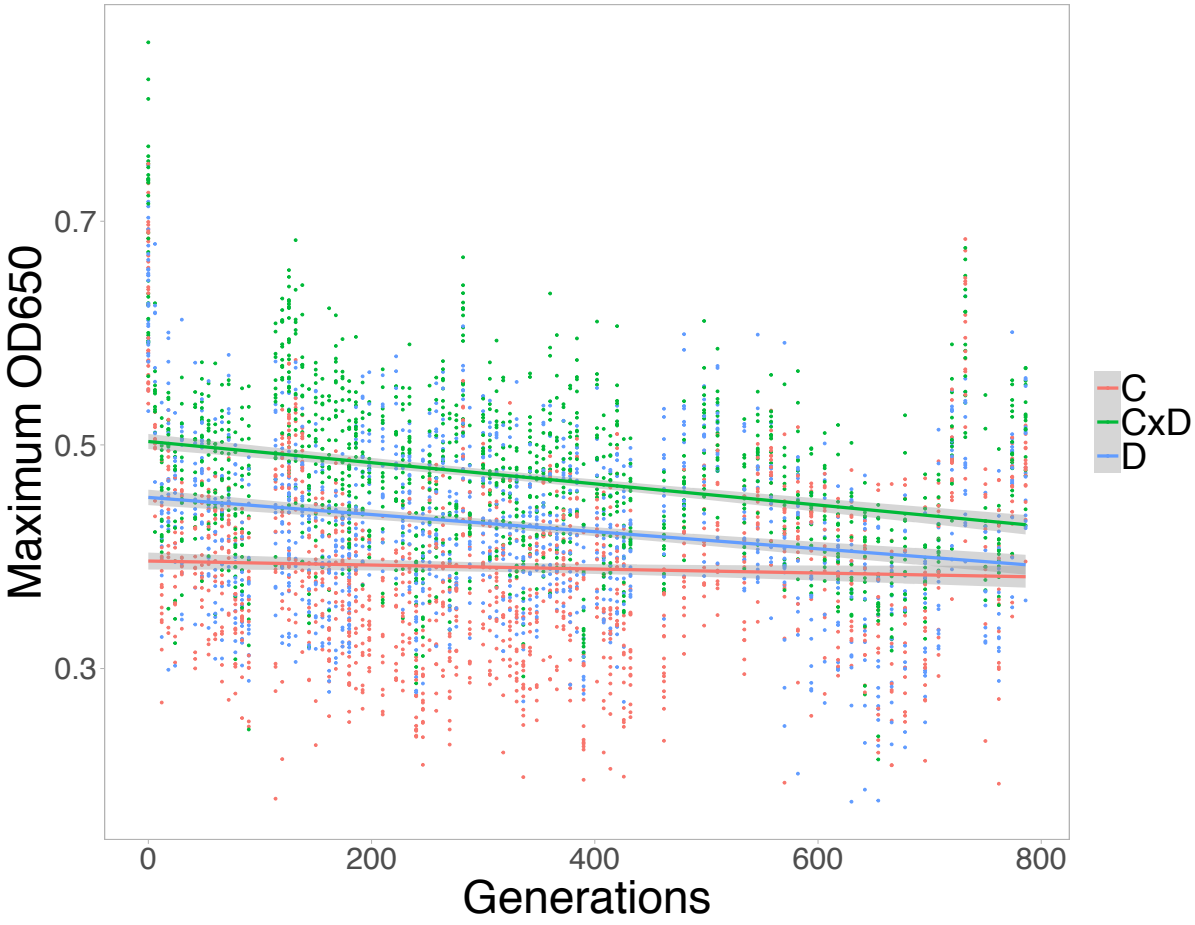

Figure 3.4. Experiment 2. Maximum OD trajectories of the parental (red and blue) and progeny (green) genotypes over nearly 800 generations of evolution. Each point is the mean growth rate per timepoint of one of the replicate populations. A linear regression with a 95% confidence interval is shown in gray.

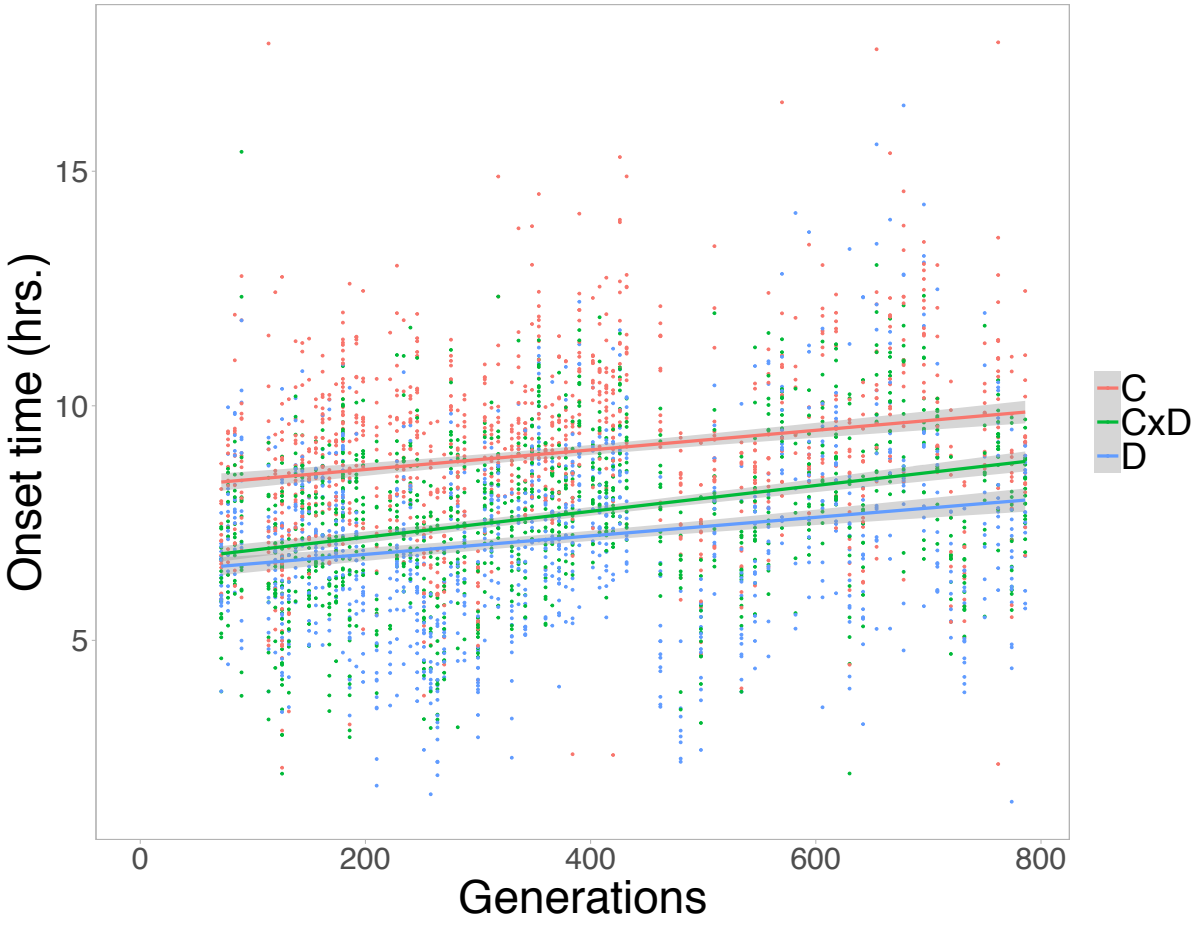

Figure 3.5. Experiment 2. Onset time trajectories of the parental (red and blue) and progeny (green) genotypes over nearly 800 generations of evolution. Each point is the mean growth rate per timepoint of one of the replicate populations. A linear regression with a 95% confidence interval is shown in gray.

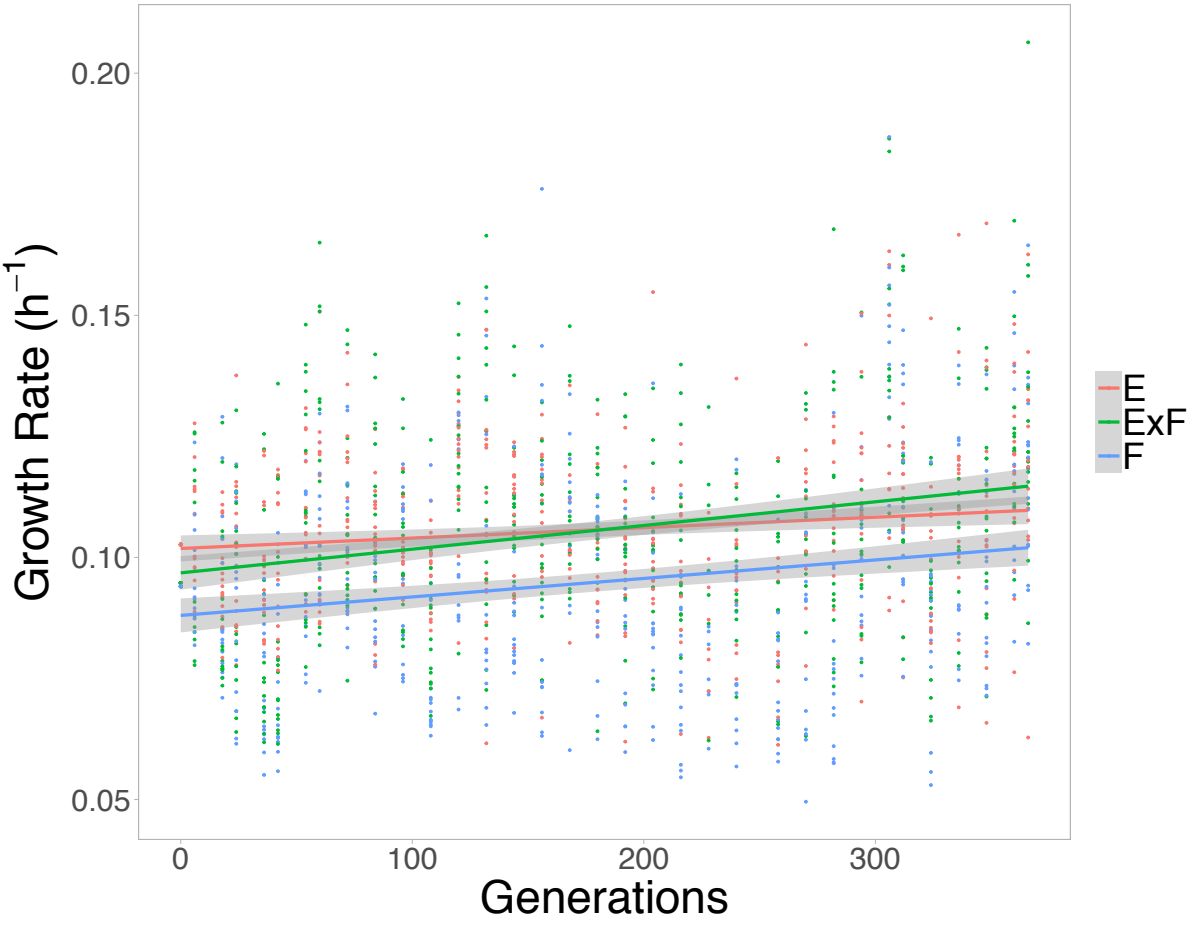

Figure 3.6. Experiment 3. Growth rate trajectories of the parental (red and blue) and progeny (green) genotypes over ~370 generations of evolution. Each point shows the growth rate from one of the replicate populations. A linear regression with a 95% confidence interval is shown in gray. The slope of the regression of the progeny populations is significantly steeper than both parents.

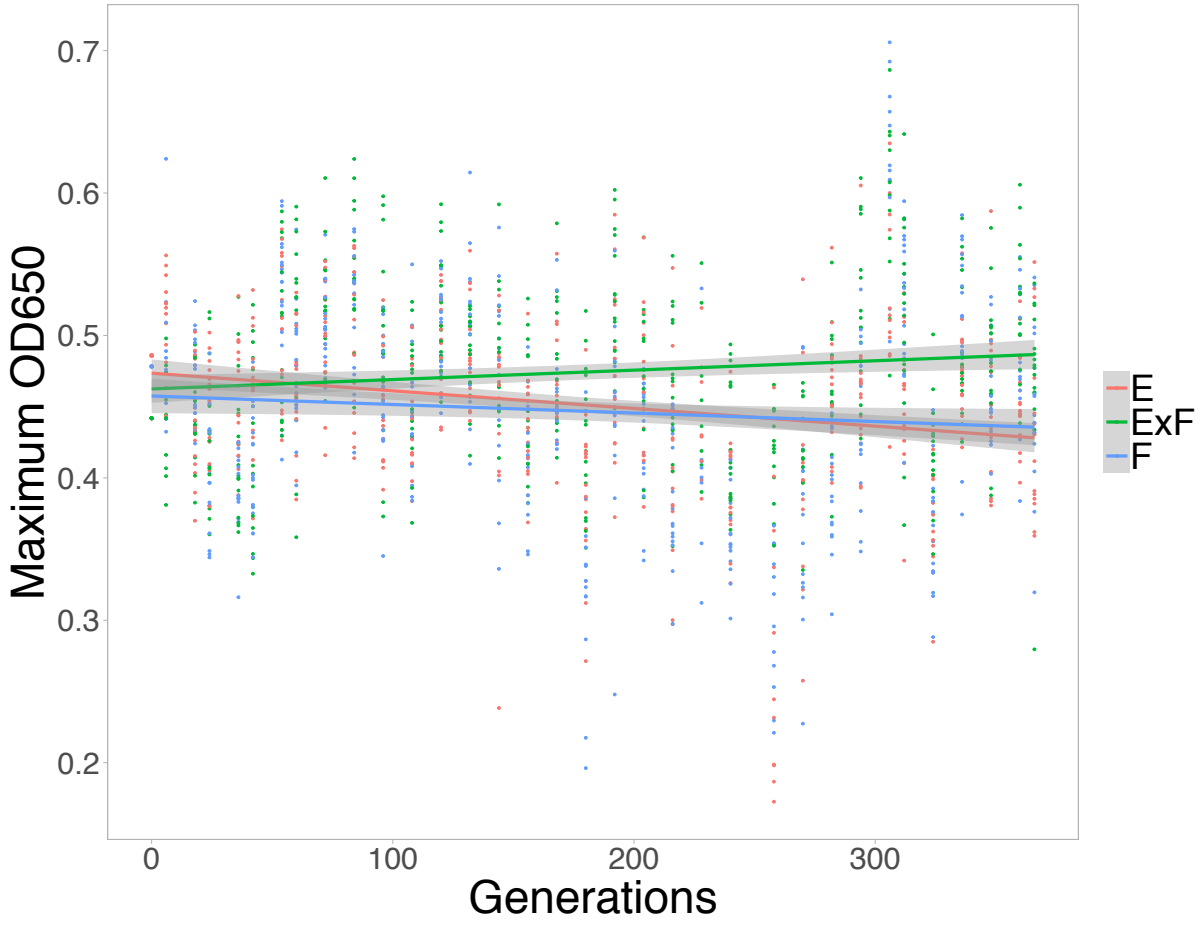

Figure 3.7. Experiment 3. Maximum OD650 trajectories of the parental (red and blue) and progeny (green) genotypes over ~370 generations of evolution. Each point shows the maximum OD650 from a growth curve of one of the replicate populations. A linear regression with a 95% confidence interval is shown in gray. The slope of the regression of the progeny populations is significantly steeper than both parents.

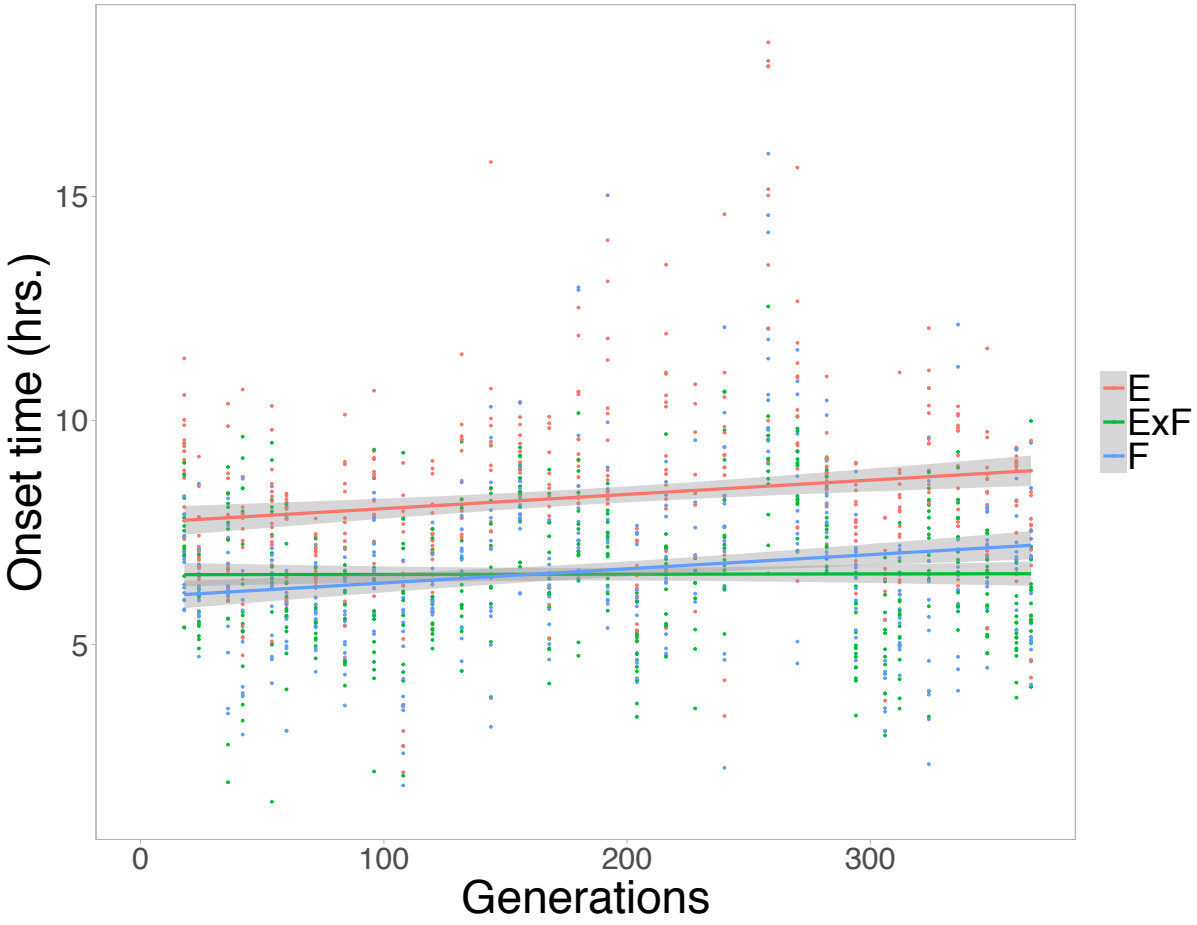

Figure 3.8. Experiment 3. Onset time trajectories of the parental (red and blue) and progeny (green) genotypes over ~370 generations of evolution. Each point shows the onset time to 0.12 OD650 from a growth curve of one of the replicate populations. A linear regression with a 95% confidence interval is shown in gray. The slope of the regression of the progeny populations is significantly less steep than both parents.

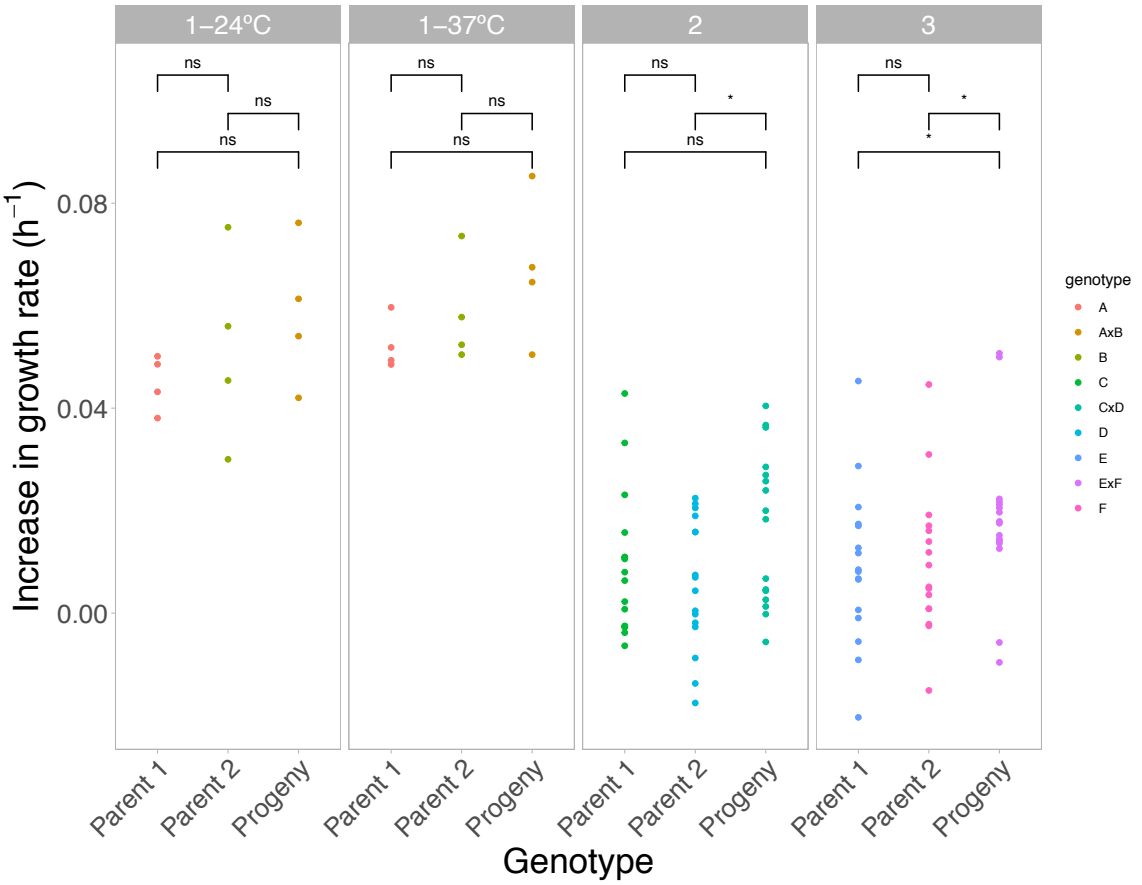

Figure 3.9. Increase in growth rate higher for sexual populations. The increase in growth rate over the course of the experiment of is shown for each population (dots). Experiment are shown separately in each panel and genotypes distinguished by color. Results of a pair-wise t-test between each genotype in the experiment are shown.

Discussion

We have demonstrated that populations of *T. thermophila* founded by a single newly produced sexual progeny are more evolvable than populations founded by their unmated parents. The unmated parents had been dividing asexually for many generations prior to the start of the experiment, and thus are likely already largely homozygous in their MAC. In contrast, the newly mated progeny should be highly heterozygous in their MAC at the start of the experiment. Thus, the increased

evolvability that we observed in the progeny is likely attributable to amitosis generating additional genetic variation in these populations. Our findings have implications for our understanding of the evolution of ciliate and *Tetrahymena* genetic architecture and the evolution of evolvability more broadly. It also improves our understanding of the advantage of sex in *Tetrahymena* in addition to explaining its persistence as an asexual.

The most widely accepted explanation for the unusual ciliate genome architecture is that genome duality evolved as a mechanism to allow foreign DNA to be sequestered in the germline (Bracht et al. 2013), and amitosis in the MAC is simply a consequence of the mechanism by which foreign DNA is eliminated from that genome. The fact that amitosis often leads to senescence and cell death in many ciliates (Simon and Orias 1987) was thus thought to be just an unfortunate side-effect of genome duality. However, we show that in *Tetrahymena* there is a period of increased evolvability following sex suggesting that amitosis can instead be beneficial. This increased evolvability thus also likely contributed to the evolutionary success of the unusual genome architecture ciliates.

Sexual reproduction is ubiquitous and ancient among eukaryotes and may be in part responsible for their massive diversification. Despite this apparent dependence on sex (at least in the long-term) amongst eukaryotes the nature of selection maintaining it is not fully understood. Importantly, these selective benefits must be quite strong to account for the various costs associated with sex (e.g., two-fold cost of sex, energetic

costs of finding a mate, breaking up beneficial combinations of alleles; Gibson et al. 2017). One of the oldest and most robust theories for the success of sex is that sex provides an indirect benefit by increasing variation in the population thereby allowing selection to operate more effectively to increase the population fitness (Weismann 1890). This hypothesis can be contrasted with the direct benefits hypothesis in which sex increases the fitness of the parent or progeny directly (Kondrashov 1993). Indirect benefits have been demonstrated in several systems. For example, sex increases the rate of adaptation in populations of *Chlamydomonas* by increasing genetic variation among offspring (Colegrave 2002; Kaltz and Bell 2002). Direct benefits have also been shown in several systems. For example in facultatively sexual species such as the ciliate *Paramecium,* which must have sex to avoid senescence (Gilley and Blackburn 1994) sex provides a direct benefit.

Here we show that in *Tetrahymena thermophila* a single sexually produced progeny has greater evolvability than either parent. This is a particularly interesting benefit of sex because although it is an indirect benefit as it takes many asexual generations and the action of selection for the benefit to manifest it is unlike the indirect benefit of sex that Weismann spoke of which requires an entire population reproducing sexually (Weismann 1890). If we consider the vegetative growth from a single sexual progeny as an individual (they share the same MIC) it can also be thought of as a direct benefit. From this perspective the direct benefit of sex is the resetting of the MAC and generation of heterozygosity, which due to amitosis increases evolvability during the vegetative growth of a single sexual progeny.

The increased evolvability following sex that we demonstrate here is not observed in non-ciliate systems. However, an analogous benefit exists when a population that reproduces primarily by selfing outcrosses generating a heterozygous progeny. Following the outcross, this heterozygosity will be subsequently lost through multiple generations of selfing during which allelic variation will be generated among the descendants of a single outcrossing event. This process resembles amitosis and phenotypic assortment but requires meiosis every generation. If the selfers are diploid, it also would happen much more quickly, with the proportion of heterozygous loci being cut in half every generation, thus ultimately generating less combinatorial variation than is possible following sex in *Tetrahymena*. This process is also fundamentally different to what happens in ciliates because the selfers are undergoing meiosis following the outcross at every generation whereas *Tetrahymena* does not. If we consider meiosis alone to be sex this benefit requires a sexual population and is akin to the indirect benefit predicted by Weismann over 100 years ago, but if we define sex to require genetic exchange among individuals then a single episode of sex could generate substantial genetic variation amongst the descendants potentially increasing their evolvability in much the same way that we observe for *Tetrahymena*.

Despite these benefits of sex, ~50% of *T. thermophila* natural isolates are asexual. In fact, some of the oldest (10 million years) well-documented cases of asexual eukaryotes are *Tetrahymena* (Doerder 2014). The increased evolvability that we

demonstrate here provides support for the hypothesis that amitosis is responsible for the success of these asexuals (Doerder 2014; Zhang et al. in submission). However, our results also suggest a reason that sexual reproduction is not lost entirely from *Tetrahymena*. By combining a dual nuclear architecture in which a somatic genome gets intermittently reset as a heterozygote with the increased evolvability associated with amitosis, *Tetrahymena* seems to be maximizing its capacity for local adaptation while minimizing the long-term risks associated with those adaptations when the environment changes. In other words, following sex they can quickly reach the highest local fitness peak but when the environment changes, making another peak higher, they can have sex and revert back to a heterozygous genotype in the valley and then quickly ascend the other peak. Without environmental change it is difficult to understand how new sexual progeny could outcompete unmated individuals that are already adapted to the environment, indicating that environmental change may play a role in maintaining sex in ciliates in the long-run (Hinton and Nowlan 1987; Watson and Szathmáry 2016).

Our results indicate that the system of inheritance*,* including the dual nuclear architecture and amitotic division, has a fundamental impact on the evolvability of *Tetrahymena.* This raises the intriguing possibility that this unique system of inheritance has itself evolved to maximize evolvability in the short-term while avoiding the long-term trade-offs associated with those adaptations when the environment changes. The two systems of inheritance (MIC and MAC) appear to respond to selection over different time-frames, with the MIC specializing in long-term adaptation

and the MAC specializing in short-term adaptation. In this sense adaptive changes in the MIC should track long-term changes in the environment or involves gradual improvement across all environments while adaptive changes in the MAC should track short-term environmental changes including local adaptation following dispersal. Further complicating the picture is the possibility of epigenetic inheritance of macronuclear mutations, which introduces a third avenue of inheritance whereby adaptations in the MAC could be passed to the new MAC for a period of time or even indefinitely as is the case with excised sequences (Eisen et al. 2006). This could allow cells to respond to environmental change over intermediate periods of time further improving the fit between genotype and environment. Our ability to predict evolutionary outcomes, as we did in this experiment, demonstrates that evolution is often deterministic. Future studies will have to investigate the limits of this determinism.

While it is highly likely that the increased evolvability of the progeny results from the variation amitosis generates from the heterozygosity produced by sex it is also possible that sex increases evolvability independently of the variation generated by amitosis. It is possible that the parents have fixed mutations in the MAC that limit their evolvability under the experimental conditions. In this case increased evolvability results from the resetting of the MAC not the benefit of amitosis. In reality both amitosis and the resetting of the MAC likely contribute to increased evolvability following sex and the effect of each likely varies depending on the situation. The relative effect of each could be determined by crossing strains that have undergone

genomic exclusion and are homozygous in the MIC (Hai and Gorovsky 1997). These sexual progeny would have a homozygous MAC and should only benefit from increased evolvability due to resetting the MAC. Therefore, the difference in evolvability between homozygous and heterozygous sexual progeny could be used to determine the precise role of amitosis in increasing evolvability.

Chapter IV

Discussion

One of the most important questions for evolutionary biologists is how variation builds up over time to create all of the diversity observed around us. Small incremental changes in isolated populations can, given enough time, lead to major differences in the organisms that make up those populations. However, we are only beginning to understand the ways in which genotype and environment contribute to this process and to the overall repeatability of evolution. To further our understanding of the roles of environment and genotype in determining evolutionary trajectories, I evolved populations of different genotypes of the ciliate *Tetrahymena thermophila* under different environmental conditions and followed their changes in growth rate as evolution occurred.

In Chapter II, I showed that evolution is more repeatable at a hotter temperature. I allowed replicate populations to evolve at two temperatures and found less variation in growth rate among the populations evolved at the hotter temperature. This was true for all three of the ancestral genotypes used in the experiment. Additionally, I

found faster convergence between the different starting genotypes at the hotter temperature. Finally, I showed that there is an asymmetry in the correlated responses between the two temperatures, meaning that evolution at the hotter temperature increases growth rate at the colder temperature more than evolution at the colder temperature increases growth rate at the warmer temperature.

In Chapter III, I showed how the novel genetic architecture of *T. thermophila* increases the evolvability of a single sexually produced progeny and discuss the evolutionary implications of this architecture. The separation of germline from soma within a single cell is an unusual but successful evolutionary strategy as we can see from the diversity of the ciliates that employ it. In *T. thermophila* we show that it provides an additional benefit following sex because of the amitotic nature of macronuclear division. In addition to the increased evolvability due to amitosis the separation of germline and soma within a single cell could also provide increased evolvability on its own. One can imagine that the micronuclear genotype could become adapted in such a way that it has access to many fitness peaks across a variety of environments. Becoming to adapted to any one environment may have large costs in others that could drive these adaptations to extinction when the environment changes. Instead *Tetrahymena* can outsource these final potential adaptations to the macronucleus so that when the environment changes they can simply have sex and revert back to the micronuclear genotype.

These results demonstrate critical the roles of environment and genotype in determining evolutionary trajectories. While the results of this experiment are specific to *Tetrahymena thermophila* by comparing our results to similar future studies in other organisms we will be able to assess how universal any environmental effects are on evolutionary trajectories.

Future directions

Relationship between growth rate and carrying capacity

The results I describe here would benefit from further work to explore the relationship between growth rate and maximum OD. Work in yeast has found that carrying capacity is maximized at intermediate growth rates (Wei and Zhang 2019). I do not see this pattern for *Tetrahymena* in the data I have collected so far. Within an environment, growth rate and carrying capacity are positively correlated however there is a negative correlation for a given genotype between the two temperature environments. Interestingly, this negative correlation may be specific to temperature as growth rate and carrying capacity appear to be positively correlated across the other environments. I can test these patterns using data that I have already collected.

Long-term fitness trajectory

In the first Chapter, I briefly discussed the shape of the long-term fitness trajectory of the first 4000 generations at either temperature. This analysis was performed on only the first 4000 generations because the 24 ºC populations had only been evolving for this long. I can redo this analysis on the 11 surviving 37 ºC populations that have now

been evolving for over 11,000 generations. Lenski showed that *E. coli* evolved for over 60,000 generations in the same simple environment are still adapting to that simple environment and increasing in fitness (Lenski et al. 2015). This came as a surprise to many as it was thought that eventually all the possible beneficial mutations would have occurred. In our analysis over the first 4000 generation we found that fitness increases appeared to reach a plateau. However, it is possible that fitness increases were just very slow, making changes in fitness between 2000 and 4000 generations undetectable. By analyzing the next 7000 generations I may be able to detect changes in fitness or confirm that fitness has indeed stopped increasing in any perceptible way. If we confirm that our fitness trajectories are different than those of the long-term evolved *E. coli* it may be because of the difference in population size or represent some more fundamental difference between eukaryotes and prokaryotes.

Molecular mechanisms underlying adaptation

My studies on the repeatability of evolution indicate that evolution is more repeatable at hotter temperatures. However, this only looked at the phenotypic level. Other studies of repeatability in evolution have found that similar evolutionary outcomes are often underlain by similar mutations (Woods et al. 2006; Good et al. 2017). Asking this same question in my evolved populations would provide insight into whether repeatability at the phenotypic level is due to repeated mutations at the molecular level. Likewise, sequence data from the populations evolved in various environments

would allow us to determine the molecular nature of genetic correlations in fitness across environments.

Appendices

Supporting Information

Tables

	Evolved at 24 ºC		Evolved at 37 ºC	
	Assayed at 24 ºC	Assayed at 37 ºC	Assayed at 24 ºC	Assayed at 37 ºC
Genotype A	**0.064** (0.057-0.071)	**0.078** (0.073-0.084)	**0.072** (0.067-0.077)	**0.087** (0.077-0.097)
Genotype B	**0.066** (0.053-0.079)	**0.065** (0.032-0.098)	**0.061** (0.045-0.078)	**0.072** (0.060-0.085)
Genotype AxB	**0.067** (0.053-0.082)	**0.072** (0.057-0.087)	**0.063** (0.057-0.068)	**0.080** (0.071-0.089)
Overall	**0.066** (0.061-0.070)	**0.072** (0.063-0.080)	**0.065** (0.060-0.070)	**0.080** (0.074-0.086)

Table S1a. Mean increase in absolute growth rate (H^{-1}) for each genotype, evolution environment, and assay temperature with 95% confidence intervals. The mean increase of all 12 populations regardless of genotype is shown in the final row of the table.

	Evolved at 24 ºC		Evolved at 37 ºC	
	Assayed at 24 ºC	Assayed at 37 ºC	Assayed at 24 ºC	Assayed at 37 ºC
Genotype A	**254%** (226-283%)	**151%** (140-163%)	**287%** (267-308%)	**169%** (150-188%)
Genotype B	**148%** (119-178%)	**78.9** (39.0-119%)	**138%** (101-175%)	**87.8%** (72.5-103%)
Genotype AxB	**157%** (124-191%)	**93.8%** (74.4-113%)	**146** (134-159%)	**104%** (91.9-116%)
Overall	**187%** (153-220%)	**108%** (85.2-131%)	**191%** (144-237%)	**120%** (96.2-144%)

Table S1b. Mean relative increase in growth rate (H^{-1}) for each genotype, evolution environment, and assay temperature with 95% confidence intervals. The mean relative increase of all 12 populations regardless of genotype is shown in the final row of the table.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	9	0.00273	0.000303	3.8781	0.0015
genotype	2	0.000700		4.479	0.0179
evolved temperature	1	0.000177		2.262	0.1408
evolved temperature*genotype	2	0.000138		0.8801	0.423
assay temperature	1	0.00131		16.69	0.0002
genotype*assay temperature	2	0.000201		1.285	0.2883
evolved temperature*assay temperature	1	0.000209		2.667	0.1107
Error	38	0.00297	0.000078		
Corrected Total	47	0.00570			

Table S2. Factors affecting absolute increase in growth rate. Standard least squares fit of the absolute increase in growth rate of each population over 4000 generations with all factors treated as fixed effects.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	9	177206.82	19689.6	75.8655	<0.0001
genotype	2	99864.22		192.39	<0.0001
evolved temperature	1	768.83		2.96	0.0934
evolved temperature*genotype	2	1779.34		3.43	0.0428
assay temperature	1	66660.82		256.85	<0.0001
genotype*assay temperature	2	7933.99		15.28	<0.0001
evolved temperature*assay temperature	1	199.62		0.7692	0.386
Error	38	9862.28	259.5		
Corrected Total	47	187069.1			

Table S3. Factors affecting relative increase in growth rate. Standard least squares fit of the percentage increase in growth rate of each population over 4000 generations with all factors treated as fixed effects.

Population	Assay temp.	N	SSE from power law	AICc from power law model	SSE from hyperbolic model	AICc from hyperbolic model	AIC evidence ratio
A-1-24	24	220	0.0769	-1744.8	0.0805	-1734.9	7.03E-03
A-2-24	24	228	0.0565	-1886.9	0.0480	-1924.0	1.13E+08
A-3-24	24	226	0.0677	-1827.3	0.0615	-1849.2	5.69E+04
A-4-24	24	216	0.0631	-1751.8	0.0582	-1769.4	6.39E+03
AxB-1-24	24	228	0.0407	-1961.7	0.0354	-1993.3	7.57E+06
AxB-2-24	24	226	0.0430	-1930.2	0.0370	-1964.1	2.25E+07
AxB-3-24	24	228	0.0650	-1854.9	0.0586	-1878.4	1.32E+05
AxB-4-24	24	225	0.0566	-1858.6	0.0491	-1890.7	9.22E+06
B-1-24	24	229	0.0503	-1922.6	0.0432	-1957.5	3.63E+07
B-2-24	24	230	0.0528	-1921.2	0.0455	-1955.3	2.44E+07
B-3-24	24	228	0.0863	-1790.4	0.0758	-1819.9	2.49E+06
B-4-24	24	225	0.0562	-1860.1	0.0553	-1864.0	6.96E+00
A-1-37	37	425	0.1819	-3290.3	0.1681	-3324.0	2.03E+07
A-2-37	37	425	0.1439	-3390.1	0.1341	-3419.9	3.00E+06
A-3-37	37	428	0.2138	-3247.5	0.1924	-3292.7	6.60E+09
A-4-37	37	428	0.2054	-3264.7	0.1870	-3304.8	5.05E+08
AxB-1-37	37	427	0.1583	-3367.4	0.1386	-3423.9	1.86E+12
AxB-2-37	37	426	0.1699	-3328.3	0.1502	-3380.7	2.39E+11
AxB-3-37	37	423	0.1795	-3278.6	0.1668	-3309.6	5.44E+06
AxB-4-37	37	429	0.1464	-3418.6	0.1253	-3485.3	3.02E+14
B-1-37	37	429	0.1383	-3443.1	0.1303	-3468.7	3.69E+05
B-2-37	37	428	0.2484	-3183.4	0.2296	-3217.0	2.02E+07
B-3-37	37	427	0.1799	-3312.6	0.1663	-3346.1	1.91E+07
B-4-37	37	430	0.1784	-3342.5	0.1611	-3386.4	3.42E+09

Table S4. Comparison of power law (growth rate = growth rate of ancestor + (θ_2 x generations)^ θ_1) and hyperbolic model (growth rate = growth rate of ancestor + (θ_1 x generations)/(θ_2 + generations)) fits to the observed changes in growth rate over 4000 generations of evolution for each population. The AIC evidence ratio indicates that 22 of the 24 populations fit the hyperbolic model substantially better than the hyperbolic model.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	9	0.0201	0.00223	144.69	<0.0001
assay temperature	1	0.0131		847.31	<0.0001
evolved temperature	1	0.000389		25.22	<0.0001
genotype	2	0.00583		189.38	<0.0001
evolved temperature*assay temperature	1	0.0000291		1.890	0.1773
genotype*assay temperature	2	0.000264		8.576	0.0008
evolved temperature*genotype	2	0.000491		15.92	<0.0001
Error	38	0.0005854	0.000015		
Corrected Total	47	0.0206			

Table S5. Factors affecting mean population growth rate at generation 0. Standard least squares fit of mean growth rate of each population at generations 0-125 with all factors treated as fixed effects.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	9	0.0337	0.00374	13.96	<0.0001
assay temperature	1	0.0155		57.81	<0.0001
evolved temperature	1	0.00445		16.61	0.0002
genotype	2	0.00793		14.79	<0.0001
evolved temperature*assay temperature	1	0.00243		9.056	0.0046
genotype*assay temperature	2	0.0000353		0.0659	0.936
evolved temperature*genotype	2	0.00333		6.21	0.0047
Error	38	0.0102	0.000268		
Corrected Total	47	0.0439			

Table S6. Factors affecting mean population growth rate at generation 3000. Standard least squares fit of mean growth rate of each population at generations 2875-3125 with all factors treated as fixed effects.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	9	0.0213	0.00237	14.61	<0.0001
assay temperature	1	0.0174		107.36	<0.0001
evolved temperature	1	0.00137		8.42	0.0061
genotype	2	0.00112		3.44	0.0425
evolved temperature*assay temperature	1	0.000515		3.17	0.0829
genotype*assay temperature	2	0.000273		0.841	0.439
evolved temperature*genotype	2	0.000642		1.98	0.152
Error	38	0.00617	0.000162		
Corrected Total	47	0.0275			

Table S7. Factors affecting mean population growth rate at generation 4000. Standard least squares fit of mean growth rate of each population at generations 3875-4125 with all factors treated as fixed effects.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	26	0.425	0.0164	37.57	<0.0001
assay temperature	1	0.271		622.30	<0.0001
genotype	2	0.0155		1.39	0.270
population[genotype]&Random	21	0.128		13.95	<0.0001
genotype*assay temperature	2	0.00599		6.87	0.0011
Error	826	0.360	0.000436		
Corrected Total	852	0.785			

Table S8. Factors affecting mean population growth rate per plate at generation 4000. Standard least squares fit of mean population growth rate per plate between generations 3875-4125 with population nested within genotype treated as a random effect.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	26	0.131	0.00503	52.77	<0.0001
assay temperature	1	0.0816		855.65	<0.0001
genotype	2	0.0410		81.66	<0.0001
population[genotype]&Random	21	0.00532		2.65	0.0002
genotype*assay temperature	2	0.00189		9.90	<0.0001
Error	283	0.0270	0.000095		
Corrected Total	309	0.158			

Table S9. Factors affecting mean population growth rate per plate at generation 0. Standard least squares fit of mean population growth rate per plate between generations 0-125 with population nested within genotype treated as a random effect.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	17	0.131	0.00503	52.77	<0.0001
assay environment	7	11.2		7.68	<0.0001
evolution environment	10	6.50		3.13	0.0030
Error	57	11.8	0.207		
Corrected Total	74	29.9			

Table S10. Factors affecting mean relative increase in growth rate of populations evolved and assayed across a range of environmental conditions. Standard least squares fit was used in analysis.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	17	32.8	1.93	6.33	<0.0001
assay environment	7	26.0		12.2	<0.0001
evolution environment	10	7.34		2.41	0.0177
Error	59	18.0	0.305		
Corrected Total	76	50.7			

Table S11. Factors affecting mean relative increase in the maximum OD640 of populations evolved and assayed across a range of environmental conditions. Standard least squares fit was used in analysis.

Figures

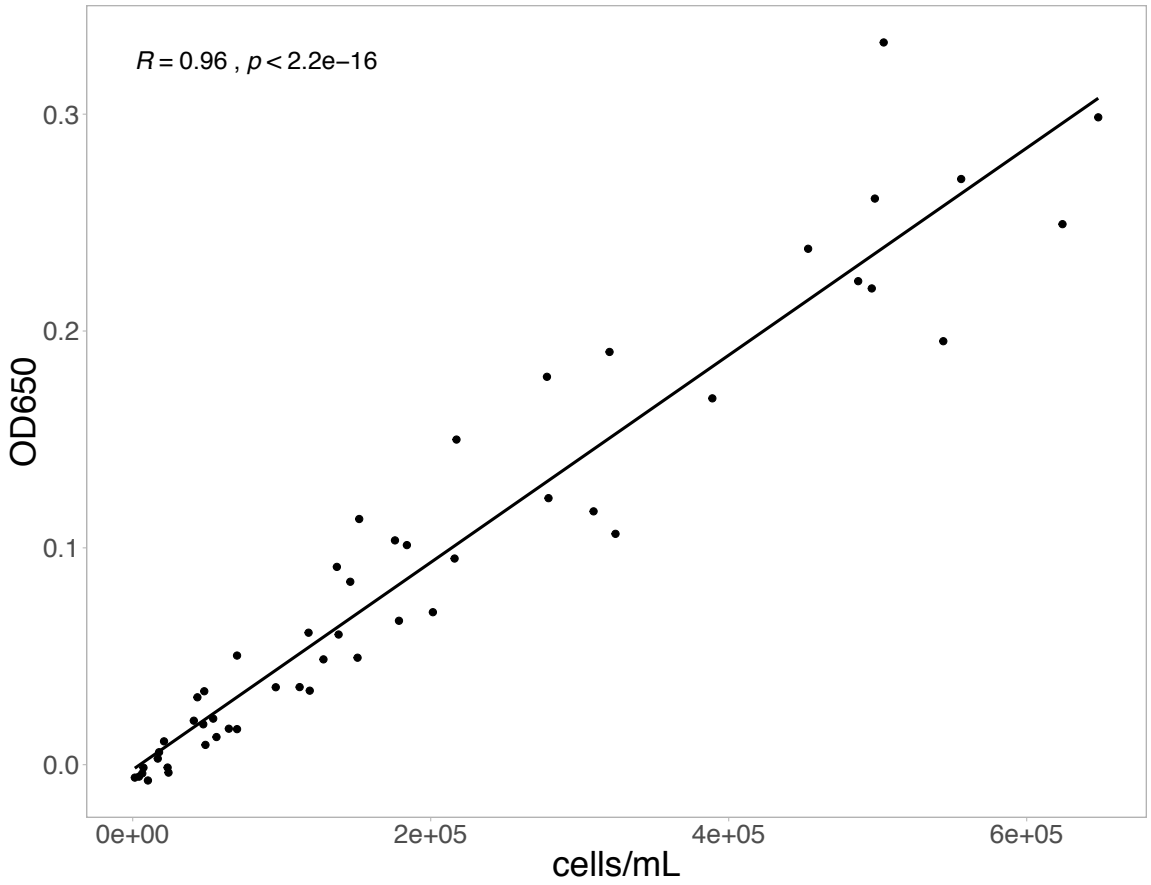

Figure S1. Correlation between OD650 and manual cell count. Each point shows the increase in OD650 attributable to cell growth and the manual cell count of a replicate population as it grows from low density to stationary phase. The black line shows the linear regression through this data. Pearson's correlation coefficient is shown in the upper left corner.

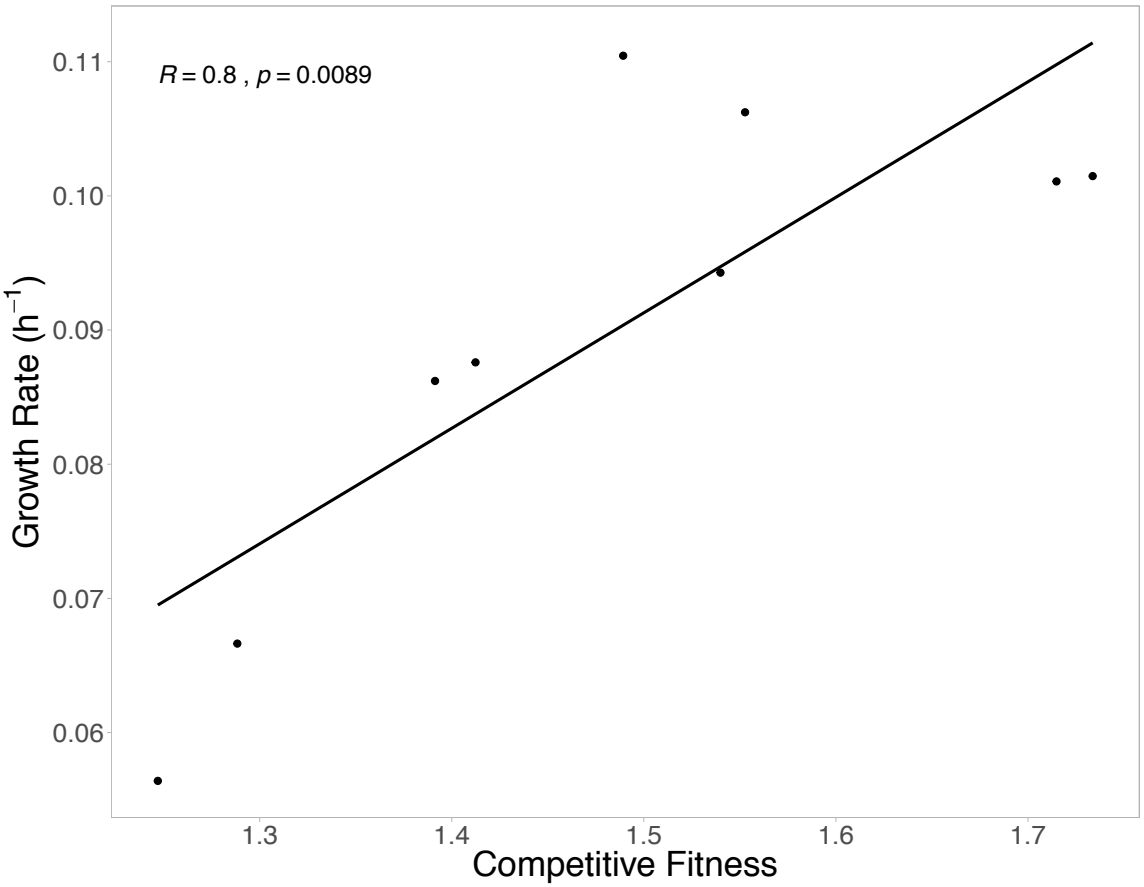

Figure S2. Correlation between growth rate and competitive fitness. Each point shows the growth rate and competitive fitness of one of nine populations for which competitive fitness was measured. The black line shows the linear regression through this data. Pearson's correlation coefficient is shown in the upper left corner.

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