

1 Temperature affects the repeatability of evolution in the microbial eukaryote

2 *Tetrahymena thermophila*

3 **Abstract**

4 Evolutionary biologists have long sought to understand what factors affect the
5 repeatability of adaptive outcomes. To better understand the role of temperature in
6 determining the repeatability of adaptive trajectories, we evolved populations of different
7 genotypes of the ciliate *Tetrahymena thermophila* at low and high temperatures and
8 followed changes in growth rate over 4,000 generations. As expected, growth rate
9 increased with a decelerating rate for all populations; however, there were differences in
10 the patterns of evolution at the two temperatures. The growth rates of the different
11 genotypes converged as evolution proceeded at both temperatures, but this
12 convergence was quicker at the higher temperature. Likewise, we found greater
13 repeatability of evolution, in terms of change in growth rate, among replicates of the
14 same genotype at the higher temperature. Finally, we found no evidence of trade-offs in
15 fitness between temperatures, but did observe asymmetry in the correlated responses,
16 whereby evolution in a high temperature increases growth rate at the lower temperature
17 significantly more than the reverse. These results demonstrate the importance of
18 temperature in determining the repeatability of evolutionary trajectories.

19

20 **Keywords:** Experimental Evolution, *Tetrahymena*, Adaptation, Repeatability,
21 Convergence, Correlated Responses, Temperature

22

23

24 **Introduction**

25 The evolutionary trajectories of both natural and experimental populations are often
26 remarkably similar to each other (Lenski and Travisano 1994; Colosimo et al. 2005;
27 Woods et al. 2006; Conte et al. 2012; Nosil et al. 2018). However, there can also be
28 substantial differences in the trajectories of initially identical experimental (Blount et al.
29 2008) and natural populations (Dieckmann and Doebeli 1999; McKinnon and Rundle
30 2002; Barluenga et al. 2006). While these types of studies have provided valuable
31 insights into the repeatability of evolutionary trajectories, we still lack a comprehensive
32 understanding of what conditions are likely to constrain trajectories from diverging due
33 to stochastic forces, and thus contribute to the repeatability of evolution.

34

35 Previous work has demonstrated that temperature can fundamentally alter evolutionary
36 outcomes, for example by increasing biological diversity at lower latitudes (Roy et al.
37 2002; Gillooly et al. 2004; Allen et al. 2006). One purported explanation for the effect of
38 temperature is that mutation rates are different at different temperatures. However,
39 empirical results are mixed, with some results showing higher mutation rates at higher
40 temperatures, others lower rates at higher temperatures, and yet others are
41 inconclusive (Faberge and Beale 1942; Kiritani 1959; Lindgren 1972). The “hotter is
42 better” hypothesis predicts that warm-adapted populations will have higher maximum
43 performance than their cold-adapted counterparts because of the evolution of greater
44 robustness due to the inherently higher rates of biochemical reactions at higher
45 temperatures (Huey and Bennett 1987; Angilletta et al. 2010). Evidence from
46 comparative and experimental populations largely supports this hypothesis (e.g., Knies

47 et al. 2009), however, again, some results are mixed (reviewed in Angilletta et al. 2010).
48 Evidence from lab evolved *Escherichia coli* shows that greater fitness gains occur at
49 higher temperatures and that populations evolved at lower temperature show trade-offs
50 at higher temperatures but not vice-versa (Bennett and Lenski 1993; Mongold et al.
51 1996). Later work suggested that while the genetic changes underlying temperature
52 adaptation were temperature specific, these mutations were also beneficial across all
53 temperatures (Deatherage et al. 2017), demonstrating that at least for the most relevant
54 mutations the observed trade-offs are not due to antagonistic pleiotropy. While trade-
55 offs could still result from the cumulative effect of less impactful mutations that show
56 antagonistic pleiotropy it is striking that the most impactful mutations do not show any
57 antagonistic pleiotropy and suggests the trade-offs, in part, result from mutation
58 accumulation at sites that are relevant at the alternative temperature but neutral at the
59 evolution temperature. Overall these results demonstrate that temperature
60 fundamentally affects adaptive outcomes, yet it remains unknown whether the
61 temperature at which a population evolves will also affect the repeatability of adaptive
62 trajectories.

63

64 To assess how temperature affects the repeatability of evolution, we performed a long-
65 term evolution experiment using the microbial eukaryote *Tetrahymena thermophila*. *T.*
66 *thermophila* is useful as a model system due to its complex life history and
67 development, and its ease of growth and tractability in lab (Nanney 1974; Merriam and
68 Bruns 1988; Prescott 1994). The short generation time and small cell size mean that
69 large populations can be evolved over many generations in the lab, and population size

70 and growth rate are easily monitored. In addition, in contrast to most other microbes in
71 which experimental evolution is regularly performed, it has a complex life history and
72 genome structure (Nanney 1974; Merriam and Bruns 1988), allowing us to test whether
73 the general patterns found in other microbes also apply to ciliates.

74

75 *T. thermophila*, like all ciliates, is notable for its genome structure. Two types of nuclei
76 are maintained in each cell. The germline micronucleus (MIC) is diploid and
77 transcriptionally silent during growth and asexual reproduction, while the somatic
78 macronucleus (MAC) is 45-ploid and transcriptionally active, meaning it gives rise to the
79 phenotype of the cell (Merriam and Bruns 1988). Ciliates are facultatively sexual,
80 mostly reproducing asexually, but occasionally undergoing conjugative sex with cells of
81 a different mating type (Nanney 1974). In our experiment, populations contained a
82 single mating type, effectively preventing sex. Thus, only mutations that occurred in the
83 MAC were subject to selection and captured in our fitness assays.

84

85 Two features of the *T. thermophila* genome may potentially impact the patterns of
86 adaptive evolution. First, the polyploid MAC divides by amitosis, a process that results
87 in the random distribution of alleles among daughter cells. Unlike with division by
88 mitosis, amitosis results in allelic variation among asexual progeny (Doerder et al.
89 1992), which generates higher levels of genetic variation and potentially increases the
90 rate of evolution. Second, *Tetrahymena* has an exceptionally low base-substitution
91 mutation rate (Long et al. 2016), which has the potential to slow the rate of adaptation.

92 However, the deleterious mutation rate is comparable to other species (Long et al.
93 2013), so the potential effect of mutation rate is currently unclear.

94

95 In this study, we conducted a long-term evolution experiment to determine how
96 temperature affects repeatability of evolution in a ciliate. We evolved populations of
97 different genotypes of *T. thermophila* in two different temperatures and monitored the
98 fitness trajectories of replicate populations. To assess the effects of temperature on the
99 dynamics of evolutionary trajectories, we asked: 1) Does the temperature at which
100 populations evolve affect the future convergence or continued divergence of initial
101 historical differences between genotypes, 2) Does evolution temperature affect the
102 repeatability of fitness trajectories, and 3) How temperature-specific are adaptations,
103 i.e., are there trade-offs or other correlated responses between temperatures? We
104 predict that temperature plays an important role in way that variation is generated and
105 acted on by selection. Thus, we expect that temperature will affect both the rate at
106 which populations converge and the repeatability of evolution. Given prior results on
107 trade-offs, we predict the populations evolving at a lower temperature are more likely to
108 experience trade-offs.

109

110 We find that populations that evolved at the higher temperature tended to have higher
111 fitness gains than their colder-evolved counterparts. The higher evolution temperature
112 also led to faster convergence among populations started from different genotypes, and
113 less divergence among replicate populations of a single starting genotype, indicating
114 that evolution at the higher temperature does indeed result in more repeatable

115 evolution. Finally, we found no indication of trade-offs, but rather an asymmetry in the
116 correlated responses, whereby evolution at the higher temperature increases fitness at
117 the lower temperature more than the reverse, possibly indicating greater environmental
118 specificity of adaptations at the lower temperature.

119

120 **Methods**

121 Summary

122 We evolved 12 populations each at both 24°C and 37°C. Each set of 12 populations
123 consisted of four replicate populations of three initial genotypes: two independent
124 natural isolates and a hybrid progeny of these two isolates. Throughout the course of
125 4,000 generations of evolution, we measured growth rate at both 24°C and 37°C for
126 each population.

127

128 Strains and initial cross

129 Natural isolates of *T. thermophila*, designated 19617-1 (Tetrahymena Stock Center ID
130 SD03089) and 19625-2 (Doerder 2019), were thawed from frozen stocks, inoculated
131 into 5.5 mL of the nutrient rich medium SSP (Gorovsky et al. 1975) in a 50 mL conical
132 tube, and incubated at 30°C with mixing for two days. These cultures were maintained
133 as the parental lines. Eight populations were established for each genotype in 10 mL
134 cultures in SSP. Four of these were maintained at 24°C and four at 37°C. These
135 populations were designated by genotype (19617-1 or 19625-2, herein referred to as A
136 and B, respectively) – replicate (1-4) – and evolution temperature (24°C or 37°C), e.g.
137 A-1-37.

138 To generate the hybrid genotype from these strains, a conical tube of each parental
139 genotype was centrifuged and the supernatant was poured off before the cells were re-
140 suspended in 10 μ M Tris buffer (Bruns and Brussard 1974). After mixing at 30°C in Tris
141 for two days to starve the cells and induce sexual competence, 1 mL of each starved
142 parental population and an additional 1 ml of 10 μ M Tris buffer were added to one well
143 in a six-well plate and placed back in the 30°C incubator. The next morning (~12 hours
144 later) the plate was checked for pairs and put back in the incubator for an additional 4
145 hours to allow progression of conjugation. Individual mating pairs were isolated under a
146 microscope using a 2 μ L- micropipette and placed in 180 μ L of SSP in one well of a 96-
147 well plate. The plate was then incubated for 48 hours after which time a single cell was
148 isolated from each well and re-cultured into 180 μ L of fresh SSP in a new well. After
149 another 48 hours at 30°C four individual cells were isolated from one of the wells, into
150 new wells with SSP, one for each of the replicate populations, and incubated at 30 °C
151 for 48 hours. Each of the four 180 μ L cultures was then split in two with each half being
152 added to a separate 50 mL conical tube containing 10 mL of SSP, one designated for
153 evolution at 37°C and the other at 24°C. These eight cultures are the starting hybrid
154 populations and are designated as A×B (19625×19617) – replicate (1-4) – evolution
155 temperature.

156

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

160

161 *Transfer regime*

162 Approximately 25,000 cells (~90 μ L) from each 37°C culture and 60,000 cells (~1 mL)
163 from each 24°C culture were transferred to 10 mL of fresh SSP daily. Transfer volumes
164 were adjusted as needed to maintain the same starting culture density at each transfer.
165 On average, the 37°C evolved populations achieved ~6.8 generations per day and the
166 24°C populations achieved ~3.5 generations per day. This means that 37°C evolved
167 populations experienced a wider range of densities during growth (~2,500 cells/mL –
168 ~275,000 cells/mL) than the 24°C evolved populations (~6,000 cells/ mL – ~60,000
169 cells/mL), starting with a lower density and ending at a higher density. We estimate the
170 effective population size to be approximately 100,000 cells for each evolved
171 environment by calculating the harmonic mean of the population size at each discrete
172 generation (Karlin 1968). To date, the 37°C populations have undergone ~9,000
173 generations of evolution and the 24°C populations have undergone ~4,000 generations
174 of evolution. Here we describe the changes in growth rate over the first 4,000
175 generations of evolution at each temperature.

176

177 *Growth curves and analysis*

178 As evolution progressed, growth rates of each population were measured at both 37°C
179 and at 24°C, i.e. at both the temperature at which they evolved and the alternate
180 temperature, on average every ~10-30 generations. Variation in number of generations
181 between measurements arose because we could not perform 37°C and 24°C assays on
182 the same days and the assays took different lengths of time at each temperature, thus
183 we would do two consecutive single days of 37°C assays, followed by a single 24°C

184 assay that lasted 2 days. Growth rate was measured by inoculating ~500 – 1000 cells
185 into one well of a 96-well plate and measuring the optical density (OD) at 650 nm in a
186 micro-plate reader every 5 minutes over the course of 24 – 48 hours for 37°C assays
187 and 48 – 72 hours for 24°C assays (see below for validation of use of OD₆₅₀ as a proxy
188 for cell density). The maximum growth rate was then estimated for each well by fitting a
189 linear regression to the steepest part of the growth curve (with OD on a log scale),
190 estimating the maximum doublings per hour (h⁻¹) (Wang et al. 2012; Long et al. 2013).
191 3 – 4 replicates of all populations were measured on a plate at each time point. ~375
192 plates containing 37°C evolved populations and ~625 plates containing 24°C evolved
193 populations were run providing approximately 500 – 1,000 growth curves at either
194 temperature per population over the 4,000 generations analyzed here.

195

196 Validation of optical density as proxy for cell density

197 To validate that OD accurately measures cell density over a range of densities, cells
198 from cultures growing on the micro-plate reader were counted under the microscope at
199 several points during the growth cycle. 3-4 replicate wells were inoculated and the plate
200 was run on the micro-plate reader at 37 °C. Every two to three hours, 5 µL of culture
201 was removed and at least 200 cells were counted to estimate cell density. The cells
202 were diluted as needed and then counted in 10 µL droplets containing approximately 40
203 cells. This process was independently repeated two times. The cell density measured
204 by counting was tested for correlation with the OD measured by the micro-plate reader
205 at each time point, and OD was found to be a good indicator of cell density (Pearson's
206 correlation coefficient = 0.9602; Fig. S1).

207 *Correlation of competitive fitness and growth rate*

208 Because it is not technically feasible in this system to measure competitive fitness for
209 the whole experiment, we measured the competitive fitness of a subset of the evolved
210 lineages at one time point, after ~1,000 or ~3,500 generations (for populations evolved
211 at 24°C or 37°C, respectively) and compared this fitness metric to our measurements of
212 growth rate. Competitive fitness was measured in replicate by competing a GFP labeled
213 strain (Cui et al. 2006) against the experimental strain. The two strains were mixed in
214 approximately 1:1 ratios and the density of both strains was determined using a flow-
215 cytometer. The culture was allowed to grow overnight at room temperature after which
216 time the flow-cytometer was used again to measure the ratio of the two strains.
217 Competitive fitness was calculated by dividing the natural log of the ratio of the final
218 population density to the initial population density of one strain by the natural log of the
219 ratio of the final population density to the initial population density of the other strain
220 (Wiser and Lenski 2015). Competitive fitness estimates correlated with our growth rate
221 estimates (Pearson's correlation coefficient = 0.7999; Fig. S2) indicating that growth
222 rate is a good proxy for fitness.

223

224 *Data analysis*

225 ~36,000 growth curves were collected from all populations over the first 4,000
226 generations of evolution. This provided us with ~1,500 growth rate estimates per
227 population over this period, approximately half at each temperature.

228

229 A generalized additive mixed model (GAMM; see supplementary information section
230 Table S10 for more detail) was fit to the mean growth rate of each population per plate
231 assayed in the environment in which they evolved. Growth rate was fit as a function of
232 generations. Models were fit that included various combinations of the terms genotype,
233 temperature, and generations and the AICs were compared using evidence ratios ($ER =$
234 $e^{(0.5 \cdot \Delta AIC)}$) to assess the significance of terms, including pair-wise and three-way
235 interactions. The three-way interaction relates to the way differences among the
236 genotypes change differently at either temperature. In other words, are there differences
237 in the patterns of convergence or divergence among genotypes between the two
238 temperatures? We also fit a standard least square model (see supplementary material
239 Table S11 for more detail) to the same dataset to assess the effects of each of the
240 parameters used in our GAMM fit.

241

242 We fit hyperbolic, power law, and linear models to the growth rate trajectories of all
243 populations assayed in the environment in which it evolved (model details are in Table
244 S4). This analysis was performed on the mean growth rate of each population per plate.
245 We computed the AICc of each fit and calculated the evidence ratio ($ER = e^{(0.5 \cdot \Delta AIC)}$)
246 to determine which model (hyperbolic, power law, or linear) best fit the trajectory.

247

248 To assess specific time points, as well as for simplicity in visualization, growth rate data
249 were also binned into 250-generation intervals (generation 0 = 0-125, generation 250 =
250 125-375, generation 500 = 375-625, etc.) and the mean growth rate at both
251 temperatures for each population was calculated. For each population the bin with the

252 highest growth rate for either temperature was identified and the absolute (i.e.,
253 maximum mean population growth rate in a 250-generation bin minus the growth rate of
254 the ancestor of that population) and the mean relative increase (i.e., (absolute
255 increase/ancestral growth rate) x 100) in growth rate was calculated from this. ANOVAs
256 testing the effects of genotype, assay temperature, and evolution temperature were
257 performed on these data (*absolute increase/relative increase in growth rate ~ genotype,*
258 *assay temperature, evolution temperature, genotype*assay temperature,*
259 *genotype*evolution temperature, assay temperature*evolution temperature*; Tables S2
260 and S3). For each ANOVA, the residuals were checked for heteroscedasticity both
261 visually and by regression analysis and none was detected. ANOVAs were also
262 performed separately on the 48 data points (24 populations x 2 assay temperatures) in
263 each bin to test for the effect of assay temperature, evolved temperature, genotype, and
264 their interactions as evolution progressed (*mean population growth rate in 250-*
265 *generation bin ~ genotype, assay temperature, evolution temperature, genotype*assay*
266 *temperature, genotype*evolution temperature, assay temperature*evolution*
267 *temperature*; Tables S5, S6, and S7). A Wilcoxon test was also used to test for
268 significant differences between genotypes (Fig. 3)

269
270 To test for significant differences at specific time points among populations evolved from
271 a single ancestor nested ANOVAs were performed on the binned data. This analysis
272 (*mean growth rate/plate ~ genotype, replicate population[genotype]&Random, assay*
273 *temperature, genotype*assay temperature*; Table S8 and S9) tested the effects of
274 replicate population treated as a random effect and nested within genotype, genotype,

275 assay temperature, and the interaction between genotype and assay temperature on
276 the mean growth rate of each population per plate. To test for differences in the
277 variance among replicate populations between evolution temperatures, ANOVAs were
278 performed separately for each evolution temperature. This analysis (*mean population*
279 *growth rate/plate ~ genotype, replicate population[genotype]&Random*; Fig. 4) tested for
280 effects of replicate population treated as a random effect and nested within genotype
281 and genotype on the mean growth rate of each population per plate in the evolution
282 environment. From this, variance components attributable to replicate population were
283 computed to assess the amount of variation that results from differences among
284 replicate populations; the inverse of this was our measure of repeatability. The same
285 analysis was performed without nesting replicate population in genotype to assess the
286 total variance among all populations as evolution progressed (*mean population growth*
287 *rate/plate ~ population&Random*; Fig. 5). This analysis shows how the variation
288 between replicates within a genotype interacts with the variation that results from
289 differences between genotypes. At each binned time point, Levene's tests were
290 performed to assess the significance of differences between evolution temperatures in
291 the variation in growth rate generated by differences both among replicate populations
292 of a single starting genotype and among all populations regardless of genotype.

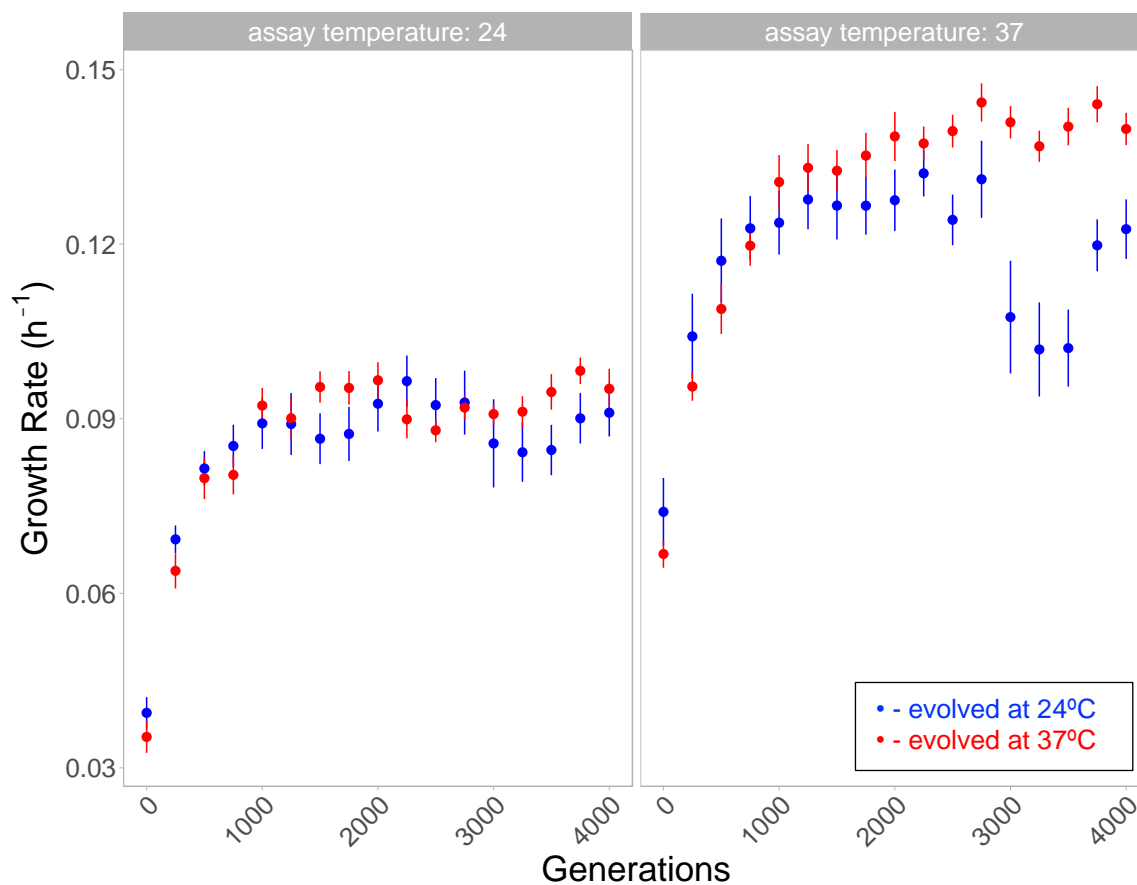
293

294 **Results**

295 *General patterns of adaptation*

296 All populations showed the expected pattern of increased growth rate over the course of
297 the experiment. The trajectories of evolving laboratory populations often follow a pattern

298 of a decelerating rate of return, characterized by larger fitness increases early in the
299 experiment, followed by incrementally smaller increases in subsequent generations
300 (Couce and Tenailon 2015; Schoustra et al. 2016; Wünsche et al. 2017). Our results
301 follow this pattern (with a linear model fitting the trajectories poorly; Table S4) and this
302 appears consistent at both temperatures (Fig. 1) and in all three genotypes when
303 analyzed separately (Fig. 2), suggesting that experimental evolution in the ciliate *T.*
304 *thermophila* does not fundamentally differ from other taxa.



305

Figure 1. Overall pattern of evolution across all populations assayed at 24°C and 37°C. Mean growth rate and 95% confidence intervals of populations evolved at 24°C (blue) and 37°C (red) when assayed at 24°C (left panel) and 37°C (right panel) are shown over 4,000 generations. Data are binned into 250 generation intervals, with the first bin containing generations 0-125.

306 Previous experiments have also shown that populations founded by initially slower
307 growing genotypes tend to increase more in growth rate over the course of an
308 experiment than those founded by initially faster growing genotypes (Jerison et al. 2017;
309 Wünsche et al. 2017). We found a qualitatively similar result whereby genotype had a
310 significant effect on the absolute increase (ANOVA: $F(2,38) = 4.48$, $P = 0.0179$; Table
311 S2) and the relative increase (ANOVA: $F(2,38) = 192.39$, $P < 0.0001$; Table S3) in
312 growth rate, and with populations founded by the slowest growing genotype (A)
313 experiencing the largest increases in growth rate for all four combinations of evolution
314 temperature and assay temperature. The mean absolute increase (i.e., the mean
315 growth rate from the highest recorded 250-generation bin minus the growth rate of the
316 ancestor of that population) and the mean relative increase (i.e., (absolute
317 increase/ancestral growth rate) x 100) in growth rate are reported for each combination
318 of genotype, evolution temperature, and assay temperature in Tables S1a and S1b. We
319 also calculated the scaled effect of parent based on the best fit model identified by
320 GAMM (see below) and found that parent A by generations was significantly positive,
321 while the scaled effect of parent B by generations and AxB by generations was
322 negative, supporting the hypothesis that the slowest growing genotype experiences the
323 greatest increase in growth rate (Table S11). However, due to the small number of
324 genotypes (3) used in this experiment we cannot definitively say this effect is due to the
325 initially lower starting growth rate of genotype A.

326

327 Unlike the long-term evolved *E. coli* lines, which continue to increase in fitness even
328 after 60,000 generations (Lenski et al. 2015), we find no significant change in mean

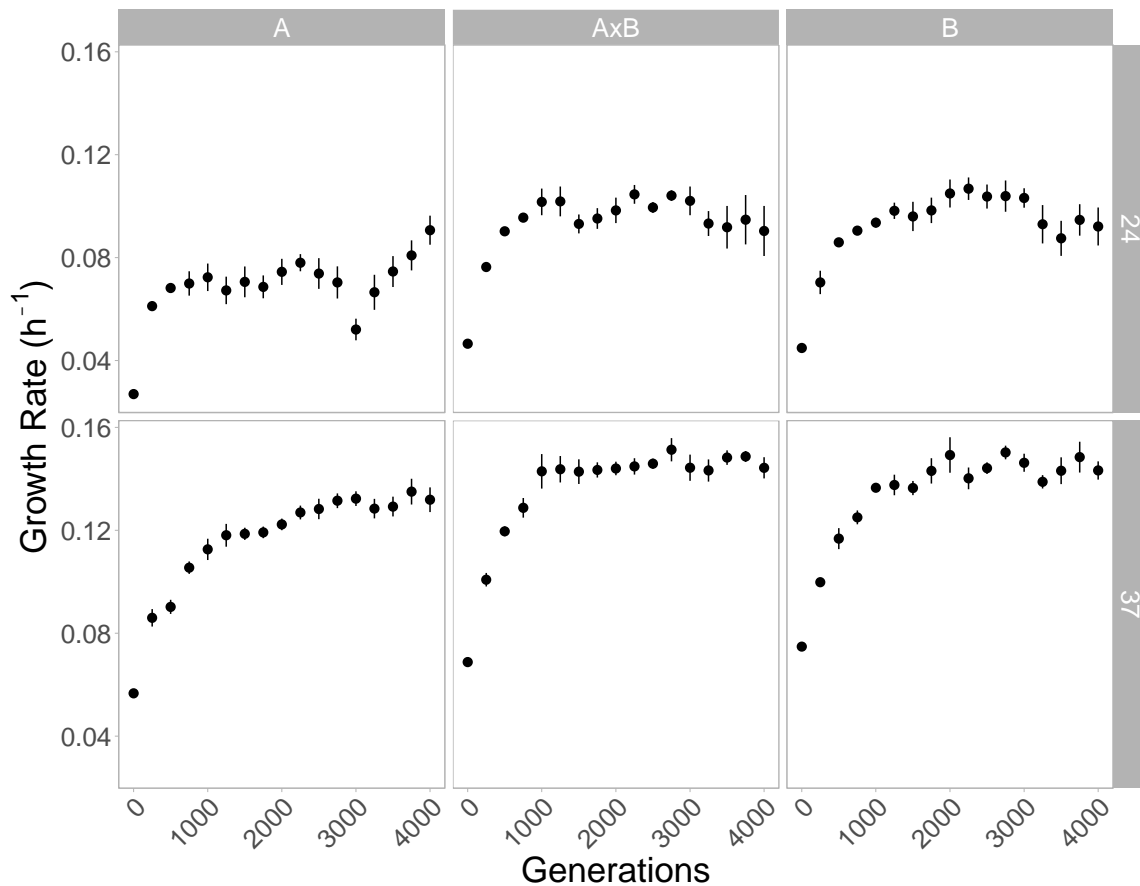


Figure 2. Fitness trajectories of each genotype assayed in their evolved temperature (correlated response at alternative temperature not shown). Mean growth rate and 95% confidence intervals of four replicate populations for each genotype are shown over 4,000 generations. The top panels show populations evolved and assayed at 24°C and the bottom shows populations evolved and assayed at 37°C. Data are binned as in Fig. 1.

329 growth rate among populations over the most recent 1,000 generations of evolution; in
330 fact our estimate of mean growth rate drops slightly from 0.1151 divisions per hour (h⁻¹)
331 at 2750 generations to 0.1130 h⁻¹ at 4,000 generations. Additionally, a hyperbolic model
332 yields a substantially better fit than a power law model or a linear model, generating a
333 significantly lower AIC value (Table S4). This suggests that the populations may have
334 reached growth rate optima upon which further improvement is unlikely. However, given
335 the limited number of generations and smaller population sizes, we are cautious in
336 interpreting this result as further evolution could lead to increases in growth rate altering

337 our model fits. It is also important to consider that fitness could be increasing in ways
338 that are not captured by our growth rate estimates so that growth rate may have
339 plateaued while fitness is still being optimized in other ways e.g., increase in carrying-
340 capacity or decrease in lag-time (Li et al. 2018).

341

342 *Evolution at a higher temperature results in faster convergence among genotypes*

343 At the start of the experiment there was a significant difference in growth rate between
344 genotypes (ANOVA: $F(2,38) = 189.38$ $P < 0.0001$; Table S5). This was true whether
345 populations were assayed at 37°C or 24°C (Wilcoxon tests; Fig. 3). Specifically, one of
346 the parental genotypes (A) grew significantly slower than the other parental genotype
347 (B) and the hybrid genotype (A×B) at both temperatures.

348

349 To determine which factors affect the evolutionary trajectories of the different
350 populations of these genotypes, we fit a GAMM and found that including the three-way
351 interaction between genotype, temperature, and generation produced the best fit with
352 the lowest AICc (see Table S10 for the models fit, the AICc of each model, and the
353 evidence ratios indicating the superior fit of the model that included the three-way
354 interaction). Based on this result, we fit a standard least square model using the same
355 terms (generations, genotype, temperature, and all interaction terms) and found that the
356 scaled effect of generations by slower growing parent (A) by 24°C was significantly
357 negative while the effect of generations by slower growing parent (A) by 37°C was
358 significantly positive (Table S11). This result indicates that genotypes are converging
359 faster at the higher temperature.

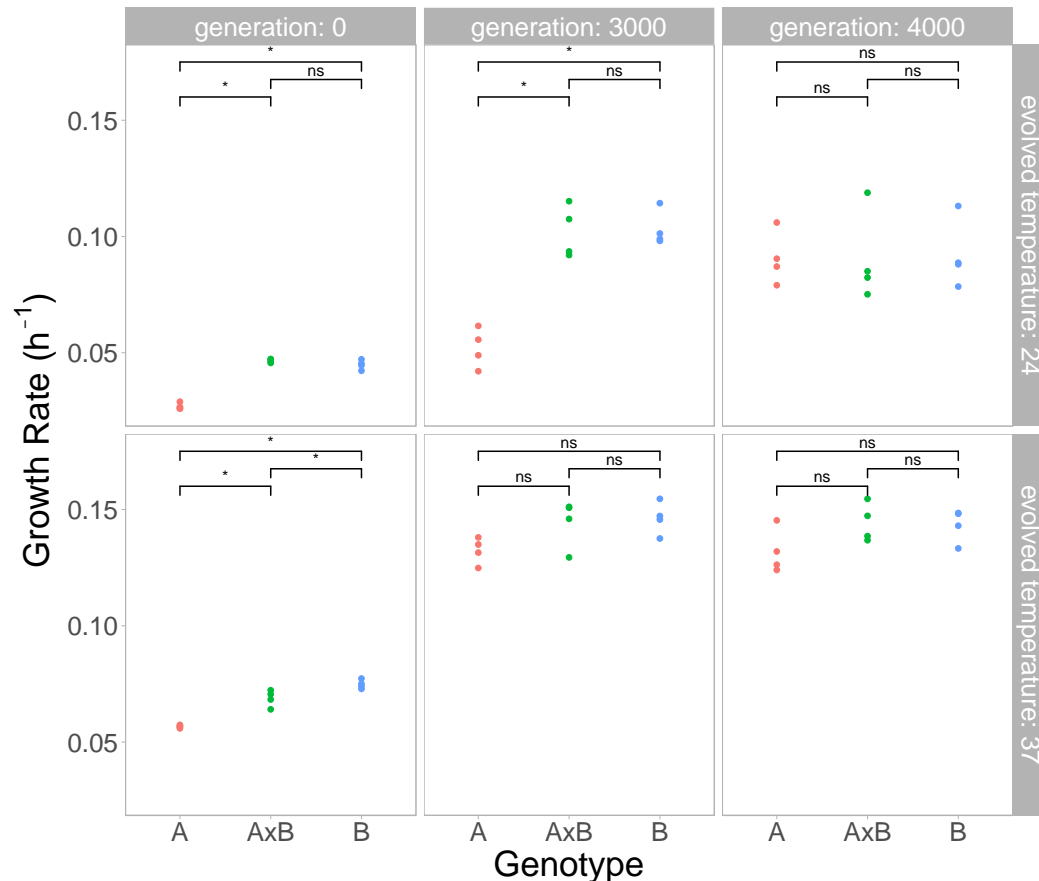


Figure 3. Genotypes converge on similar growth rates faster at the higher temperature. Differences in growth rates in the home environment (i.e. assay temperature the same as the evolution temperature) among genotypes (A = red, AxB = green, B = blue) are shown at three time points (0, 3,000, and 4,000 generations) at each temperature. Each point shows the mean growth rate of one out of the four replicate populations. A Wilcoxon test was used to determine significant differences between genotypes (“*” indicates $p < 0.05$, “ns” indicates no significant difference).

360 To further explore this result, we used ANOVA to determine at which generations there
 361 remains a significant difference between genotypes at each temperature. The difference
 362 between genotypes remained at both temperatures for nearly 3,000 generations of
 363 evolution. After 3,000 generations, we still find an effect of genotype on growth rate
 364 (ANOVA: $F(2,38) = 14.79$, $P < 0.0001$; Table S6), however after investigating the
 365 significant interaction effect of genotype by evolution environment (ANOVA: $F(2,38) =$
 366 6.21 , $P = 0.0047$; Table S6) we found this effect is driven primarily by the 24°C evolved
 367 populations at this time point. In fact, the significant difference between genotypes is

368 lost after 3000 generations of evolution at 37°C ($R^2 = 0.0301$) but not at 24°C ($R^2 =$
369 0.472; Wilcoxon test; Fig. 3), supporting the finding that the genotypes converge on a
370 similar growth rate more quickly at the higher temperature. By 4,000 generations there
371 is still a significant, but smaller effect of genotype on growth rate (ANOVA: $F(2,38) =$
372 3.44, $P = 0.0425$; Table S7) however Wilcoxon tests detect no significant differences
373 between genotypes at either temperature (Fig. 3).

374

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

376 The variation in growth rate among replicate populations appeared greater in
377 populations evolved at 24°C compared to those evolved at 37°C. To test whether
378 apparent differences between replicate populations evolved from a single ancestor were
379 significant we performed a nested ANOVA on mean growth rate per plate at 4000
380 generations. We found a significant effect of replicate population nested within genotype
381 ($F(21,826) = 13.95$, $P < 0.0001$; Table S8) indicating significant divergence between
382 populations evolved from a single ancestor. Similar results were obtained for other time
383 points. In fact, even as soon as generation 125 there is an effect of population nested
384 within genotype ($F(21,283) = 2.65$, $P = 0.0002$; Table S9) indicating that populations
385 began to evolve measurable differences in growth rate early in their evolution. To further
386 analyze this result and to assess differences in the variance produced at either
387 evolution temperature, we performed Levene's test every 250 generations and
388 compared the variance component attributable to replicate population (nested within
389 genotype) at either evolution temperature (Fig. 4). The variance component attributable
390 to population is a measure of repeatability because it describes how similar or different

391 the growth rates of replicate populations are within each genotype. We also compared
392 the variance component attributable to population regardless of genotype using an
393 unnested model for either temperature (Fig. 5). This allows us to see how the decrease
394 in variation between genotypes (Fig. 3) interacts with the variation produced among
395 replicate populations of a given genotype (Fig. 4) to affect the overall variation between
396 all populations regardless of genotype.

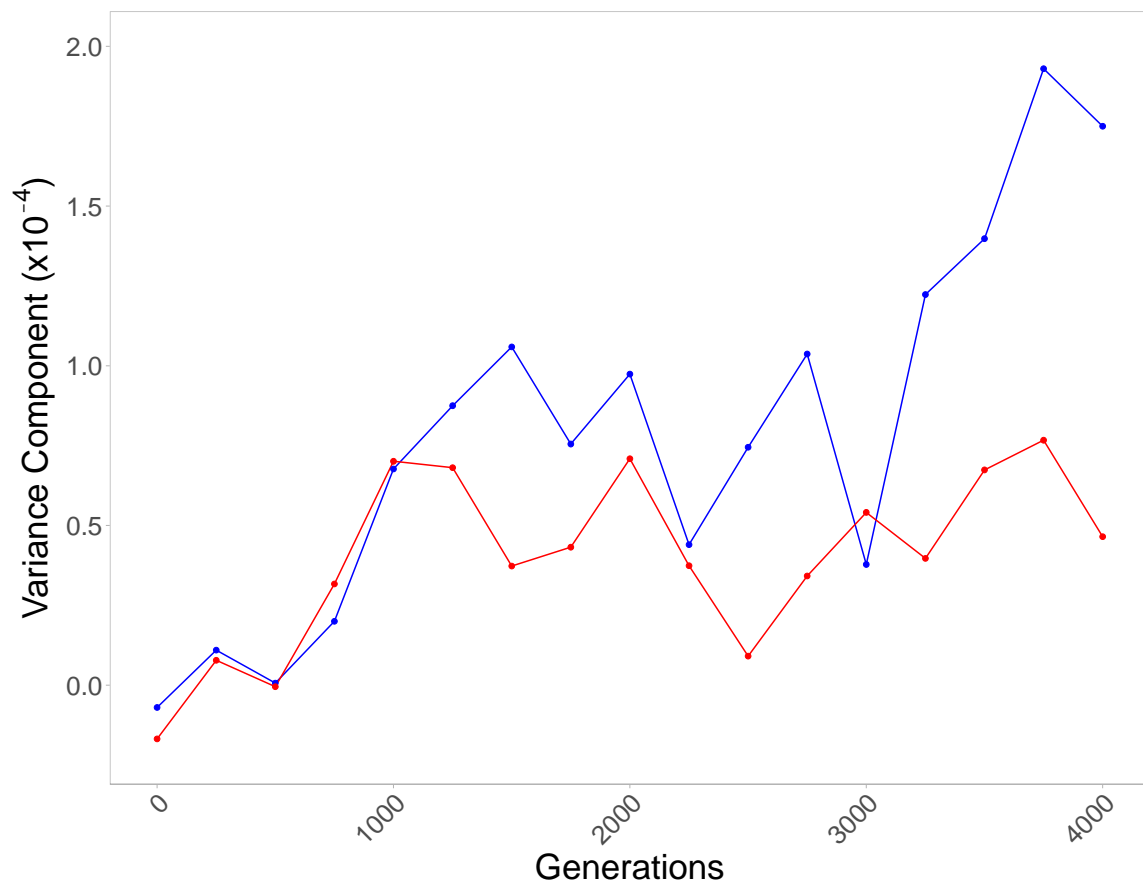
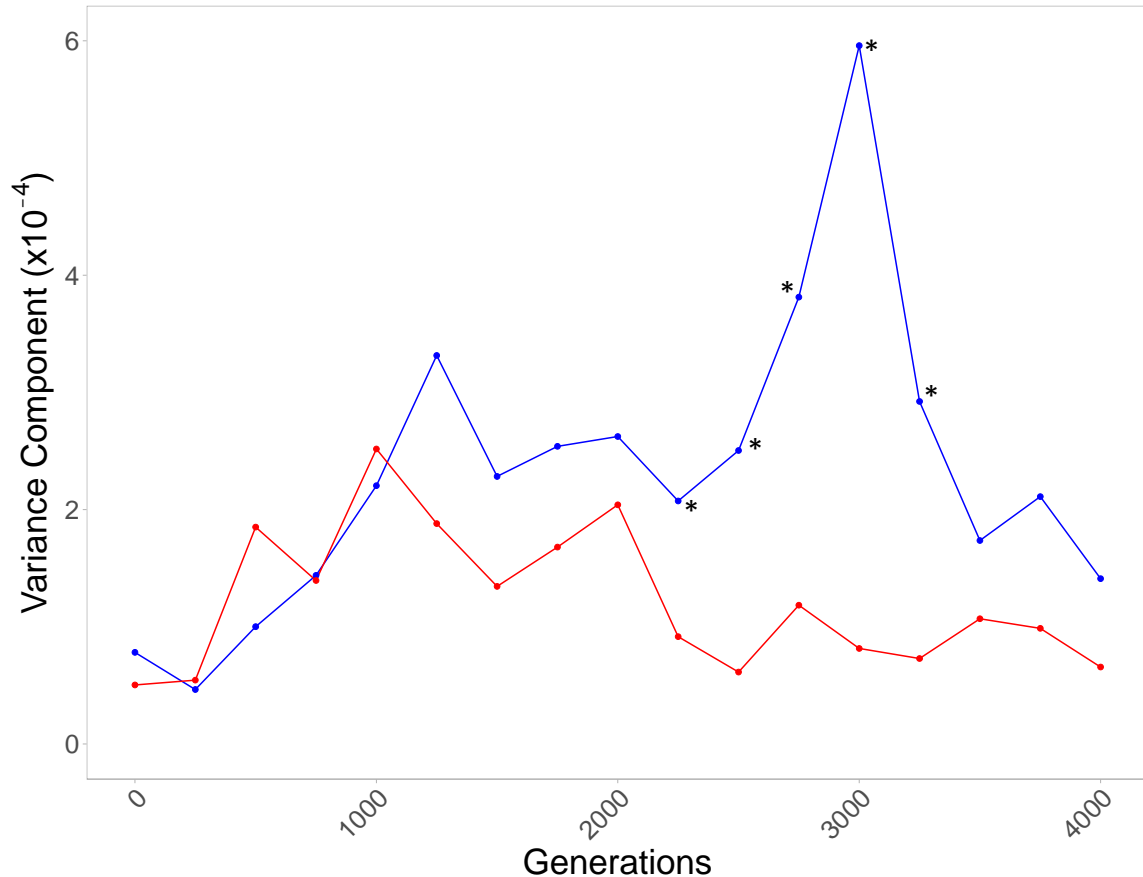


Figure 4. Variance in growth rate due to divergence among replicate populations. The variance components attributable to replicate population for populations evolved and assayed at 24°C (blue) or 37°C (red) over 4,000 generations of evolution. Variance components were estimated from an ANOVA with replicate population nested within genotype (*mean population growth rate/plate ~ genotype, replicate population[genotype]&Random*) for each 250-generation bin and evolution temperature.

397



398

Figure 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 °C (blue) or 37 °C (red) over 4000 generations of evolution. Variance components were estimated from an ANOVA without population nested within genotype (*mean population growth rate/plate ~ population&Random*) for each 250-generation bin and evolution temperature. Asterisks indicate significant results of Levene's test.

399

400 The small sample size within a genotype (n=4) meant Levene's test was unable to
401 detect significant differences in the variance between temperatures at each individual
402 time point, but we consistently see a larger variance component attributable to replicate
403 population nested within genotype among populations evolved and assayed at 24 °C
404 particularly after 1000 generations (Fig. 4). This is true regardless of assay temperature,
405 indicating that evolution temperature is likely driving this effect, and supporting our
406 hypothesis that temperature impacts the repeatability of the growth rate trajectories of
407 replicate populations.

408 When we combine growth rate data from all genotypes Levene's tests indicate there is a
409 significant difference in the variance among populations at either temperature from
410 generation 2,250 to generation 3,250 (Fig. 5). We also find consistently lower variance
411 components attributable to population among 37°C-evolved populations than those
412 evolved at 24°C (Fig. 5). This is due to the joint effect of less divergence between
413 replicate populations of the same genotype (Fig. 4) and more convergence among
414 different genotypes for populations evolved at 37°C relative to those evolved at 24°C
415 (Fig. 3). At both temperatures the variance component attributable to population peaks
416 at an intermediate generation, although the peak is higher and later for populations
417 evolved at 24°C, as variation accumulates among replicate populations but before
418 genotypes have had sufficient time to converge (Fig. 5).

419

420 In spite of the greater variation among replicate populations of the same genotype
421 evolved at 24°C (Fig. 4) we still detect greater differences among genotypes when
422 evolution takes place at 24°C (Fig. 3). This indicates that the observed differences
423 among genotypes at 24°C vs. 37°C (described in the section above) are not just due to
424 higher variability among replicate populations at the lower temperatures, but also to
425 longer lasting differences between genotypes. Additionally, the increased variance
426 among lines evolved at the colder temperature is consistent when we look at the growth
427 rate at the alternate temperature indicating this pattern is not the result of measurement
428 differences between the two temperatures and is indeed the result of the evolution
429 temperature.

430

431 *Asymmetry of the correlated responses*

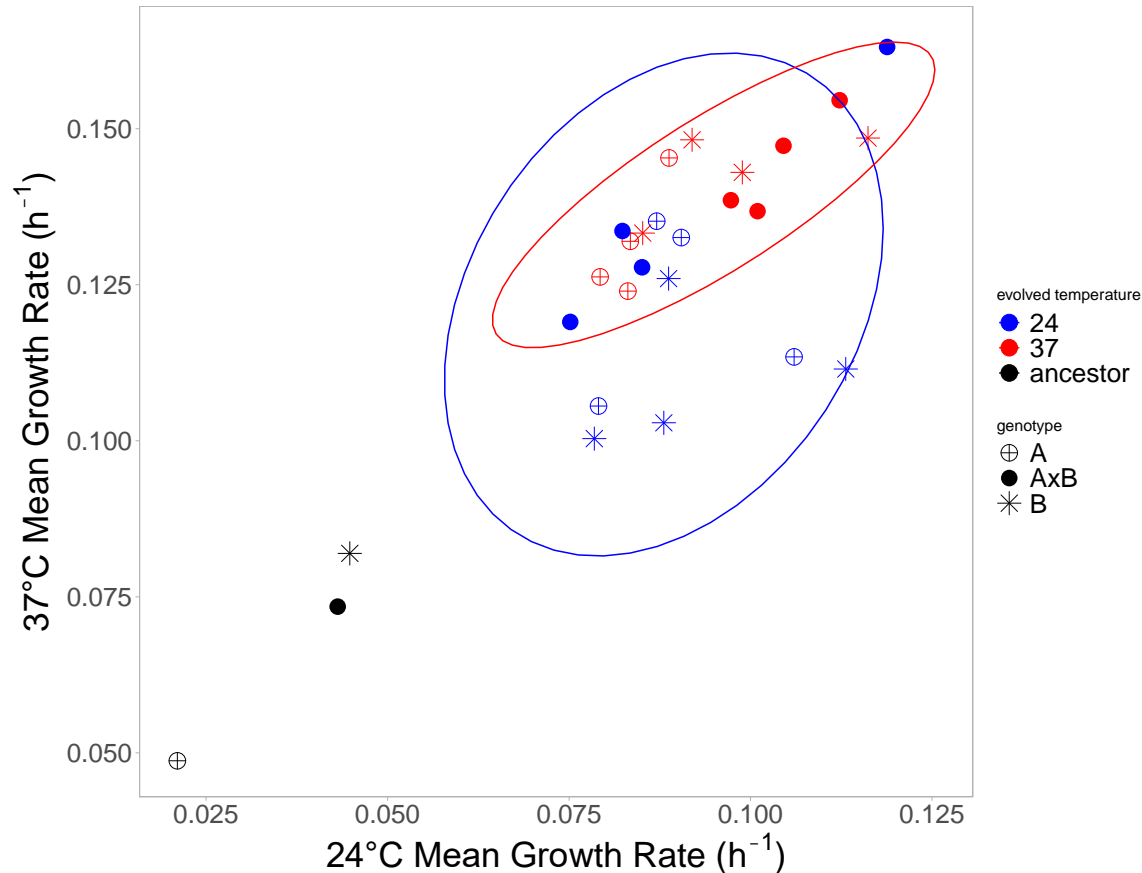


Figure 6. Correlation between growth rates in alternative environments. Growth rate of populations after 4000 generations of evolution, measured at 37°C (y-axis) or 24°C (x-axis). Genotypes are indicated by the symbols and the evolution environment is indicated by red (37 °C) or blue (24 °C) with the 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 °C (red) and for populations evolved at 24 °C (blue).

432 By generation 4,000, all populations increased in growth rate at both the temperature in
433 which they evolved and the alternate temperature (Table S1), indicating no evidence of
434 trade-offs at this time point. However, we find a marginally significant interaction
435 between evolution temperature and assay temperature (ANOVA: $F(1,38) = 3.17$, $P =$
436 0.0829 ; Table S7) at generation 4,000. This suggests that some of the adaptation that
437 has taken place over the course of the experiment is temperature-specific despite an
438 overall correlation between growth rates of evolved populations at either temperature (r

439 = 0.597). This correlation is even greater when the ancestors are included in the
440 analysis ($r = 0.858$; Fig. 6).

441

442 To assess which temperatures were driving the interaction between evolution
443 temperature and assay temperature, we compared growth rates from each assay
444 temperature. We found a significant effect of evolution temperature when assays were
445 performed at 37°C ($R^2 = 0.285$) but, remarkably, not at 24 °C ($R^2 = 0.0265$; Tukey-
446 Kramer: $p < 0.05$). This means that even after 4,000 generations of evolution, the
447 temperature at which populations evolved makes no difference when growth rate is
448 assayed at 24 °C. This indicates there is a greater correlated response when evolution
449 occurs at 37 °C. In other words, evolution at the hotter temperature increased growth at
450 the colder temperature more than evolution at the colder temperature increased growth
451 at the hotter temperature (Fig. 6).

452

453 **Discussion**

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

460

461 After 4,000 generations, we found that populations evolved at 37 °C significantly
462 outperformed those evolved at 24 °C (Fig. 1). This outcome aligns with previous findings
463 that "hotter is better" (Knies et al. 2009; Angilletta et al. 2010). This hypothesis states
464 that hot-adapted genotypes will have higher maximum growth rates than cold-adapted
465 genotypes because they have evolved greater robustness in response to the chemical
466 and metabolic reactions happening more quickly at hotter temperatures and because
467 the rate-depressing effects of low temperature cannot be overcome by adaptation or
468 plasticity.

469

470 *Temperature affects the convergence of different genotypes*

471 Over the course of evolution, different starting genotypes and phenotypes could
472 converge, evolve in parallel, or diverge even further. Through epistatic interactions,
473 genotype can constrain the future evolution of a population by biasing the set of
474 available beneficial mutations that are likely to be selected (Draghi and Plotkin 2013).
475 Similar genotypes are expected to fix a similar set of mutations while more divergent
476 genotypes are expected to fix a less similar set of mutations leading to further
477 divergence between the genotypes (Blount et al. 2018; Starr et al. 2018). At the same
478 time natural selection could overcome both random drift and epistatic interactions to
479 produce convergence between divergent genotypes.

480

481 Previous experiments have found that the rate of adaptation is inversely proportional to
482 initial fitness and that initially different populations often end up at the same fitness
483 optima (Jerison et al. 2017; Wünsche et al. 2017). At the same time studies have also

484 found that particular alleles can impede this fitness recovery and constrain the future of
485 evolution (Woods et al. 2011; Jerison et al. 2017). However, these experiments were
486 limited to less than 1,000 generations of evolution and it is unclear whether continued
487 evolution would eventually allow these populations to reach the same fitness optimum
488 as their relatives. For more distantly related populations, we might expect this process
489 to take longer if it even occurs at all.

490

491 In our experiment, the maintenance of historical differences between divergent
492 genotypes of the same species over many generations of evolution at both
493 temperatures suggests that genetic differences in the initially slowest growing genotype
494 are impeding future adaptation in a manner that is not easily overcome. Despite the
495 overall increase in growth rate being greatest for the initially less fit genotype as
496 expected, we observe slower rates of adaptation for this genotype than we would
497 expect if all genotypes followed the same pattern of diminishing returns epistasis. We
498 also find that temperature affects this pattern and the rate of convergence. Differences
499 in growth rate between genotypes were maintained for over 3,000 generations at 24°C
500 while convergence among the genotypes was more rapid at 37°C. Why a higher
501 temperature would be more conducive to convergence is unclear but could be related to
502 other effects of temperature observed in our experiment. For example, higher selection
503 coefficients and/or more targets of selection at 37°C may contribute to the slower
504 growing genotype catching up more quickly at this temperature, to the greater
505 repeatability, and to the asymmetry of the correlated responses.

506

507 The ability of populations to escape constraints on evolutionary change can be vital to
508 long-term survival (Chao and Weinreich 2005; Weinreich et al. 2005). In this
509 experiment, we show the gradual loss of growth rate differences between genotypes
510 even while differences evolve among replicate populations of the same genotype at
511 both temperatures. This suggests that differences in patterns of divergence depend on
512 relatedness, e.g. increasing divergence among genetically identical replicates, but
513 decreasing variation among less related genotypes as the mean growth rates of
514 divergent genotypes converge in the same environment. However, very distantly related
515 genotypes may find drastically different evolutionary solutions to the same
516 environmental pressures, which could contribute to further phenotypic divergence.
517 Therefore, it is possible that phenotypic divergence is minimized at intermediate levels
518 of relatedness.

519

520 *Temperature affects repeatability among populations*

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

527

528 The greater variation among populations evolved at 24°C suggests that these
529 evolutionary trajectories are more dependent on chance events than the populations

530 evolved at 37°C. This result may reflect differences in the environment that affect the
531 degree of epistasis or “ruggedness” of the fitness landscape and/or rate of mutation and
532 distribution of their effects.

533

534 Differences in the “ruggedness” of the fitness landscape, caused by epistatic
535 interactions (Kvitek and Sherlock 2011; Poelwijk et al. 2011), at each temperature could
536 explain our observation of increased repeatability at 37°C. While theory predicts that a
537 rugged fitness landscape can increase the repeatability of evolution at the level of the
538 mutational pathways followed (De Visser and Krug 2014) the opposite is true at the
539 fitness level (Bank et al. 2016). Therefore, theory suggests, the greater repeatability in
540 growth rate (a good proxy for fitness) trajectories at 37°C could result from a more
541 uniform fitness landscape at this temperature.

542

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

553

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

564

565 *Temperature affects correlated responses*

566 Experiments using *E. coli* have found substantial evidence for temperature associated
567 trade-offs (Bennett et al. 1992; Bennett and Lenski 1993, 2007; Mongold et al. 1996;
568 Woods et al. 2006). In *T. thermophila*, we find no evidence for trade-offs in any of our
569 populations after 4000 generations. However, we do find an asymmetric correlated
570 response, whereby evolution at 37°C increases growth rate at 24°C more than evolution
571 at 24°C increases growth rate at 37°C, which is similar to what is observed in *E. coli*.
572 Evolution at a hotter temperature increases growth rate at a colder temperature for both
573 species while evolution at a colder temperature increases growth rate at a hotter
574 temperature less for *T. thermophila* and often decreases it for *E. coli* (Bennett et al.
575 1992; Bennett and Lenski 1993; Mongold et al. 1996). One likely explanation for the

576 difference between *T. thermophila* and *E. coli* is that the *E. coli* experiments started
577 from an ancestor that had already evolved under laboratory conditions for 2,000
578 generations and was therefore pre-adapted to the general culture conditions, as
579 opposed to our *T. thermophila* lines, which were derived from wild collected strains
580 grown in lab only ~500 generations before cryopreservation. Thus, it seems likely that a
581 greater proportion of the adaptation that occurred in the *T. thermophila* populations,
582 compared to the *E. coli* populations, involved adaptation to the general culture
583 conditions as opposed to the specific temperature.

584

585 As evolution occurs in one environment, fitness may change in other environments
586 either as a direct pleiotropic response to selection in the evolution environment or due to
587 the accumulation of mutations that are neutral in the evolution environment but have
588 fitness consequences in the other environment (Cooper and Lenski 2000). The
589 asymmetry we observe in the correlated responses could be due to asymmetry in the
590 pleiotropic responses, whereby a 37°C beneficial mutation increases growth rate more
591 at 24°C than a 24°C beneficial mutation does at 37°C. Alternatively, the asymmetry in
592 the correlated responses could arise from an asymmetry in the effect of neutral and
593 nearly neutral mutations at the alternate temperature. In other words, the neutral and
594 nearly neutral mutations that are able to accumulate at 37°C are also mostly neutral at
595 24°C while the neutral and nearly neutral mutations that are able to accumulate at 24°C
596 tend, on average, to be slightly deleterious at 37°C. These two possibilities are not
597 mutually exclusive.

598

599 One possible mechanistic explanation for the observed asymmetry could be more
600 transcript diversity, and thus more targets of selection, in hotter conditions resulting in
601 most genes that are transcribed and selected at 24°C also being transcribed and
602 selected at 37°C but not vice versa. This would be consistent with the lack of
603 antagonistic pleiotropy across temperatures among the most positively selected
604 mutations found in lab-evolved *E. coli* (Deatherage et al. 2017) and is supported by data
605 showing that more genes are up-regulated at hotter temperatures (Tai et al. 2007; Mittal
606 et al. 2009). Additionally, the 37°C evolved populations divide more quickly and
607 experience a greater density range, and thus a more heterogenous environment, than
608 those evolved at 24°C, which could also contribute to greater transcript diversity and the
609 asymmetry in the correlated response that we observe. This idea is supported by a
610 meta-analysis of trade-off experiments, which found that populations evolved in
611 homogeneous environments exhibited more trade-offs than populations evolved in
612 temporally heterogeneous environments (Bono et al. 2017). However theoretical
613 predictions made by Gilchrist (1995) suggest, somewhat counterintuitively, that the
614 opposite should be true and that temporal heterogeneity should lead to greater thermal
615 specialization. The 37°C populations also experience an additional possible source of
616 heterogeneity because the 37°C tubes are not pre-heated so the cells experience the
617 24°C temperatures for a very brief period each day. It is conceivable that this very brief
618 period of cold is sufficient to explain the greater correlated response in the 37 °C
619 evolved populations. However, we consider this unlikely as this cold exposure is taking
620 place during lag phase, not when cells are dividing, and is therefore unlikely to impact
621 selection on the growth rate.

622

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

637

638 Another possible interpretation of our results is that populations evolving at 24°C adapt
639 by increasing different components of fitness than those evolving at 37°C. We
640 measured growth rate, which is a major component of fitness, and well correlated with
641 competitive ability in our experiments, but fitness can also increase in more complex
642 ways than simply increasing maximum growth rate (Li et al. 2018). For example,
643 decreasing lag time or increasing carrying capacity could increase fitness without
644 affecting growth rate. Additionally fitness gains can be accrued and realized in different

645 portions of the growth-cycle (Li et al. 2018), which could contribute to the asymmetry of
646 the correlated responses that we observe if the amount of time spent in different phases
647 of the growth cycle differs substantially between temperatures. A final caveat is that all
648 of the adaptation that we observed occurred in the somatic nucleus, which is discarded
649 following sexual reproduction. While there is evidence of some epigenetic inheritance
650 between parental and progeny somatic genomes (Beisson and Sonneborn 1965;
651 Chalker and Yao 1996; Pilling et al. 2017), it is unknown whether any of the adaptation
652 that occurred in our experimental populations would be inherited by newly produced
653 sexual progeny. However, this may be a moot point in this experiment because all of the
654 evolved populations lost the ability to undergo sexual conjugation, at least under
655 laboratory conditions.

656

657 *Conclusion*

658 One of the most important questions for evolutionary biologists is how variation builds
659 up over time to create all of the diversity observed around us. Small incremental
660 changes in isolated populations can, given enough time, lead to major differences in the
661 organisms that make up those populations. However, selection can also result in
662 striking examples of parallel and convergent evolution and we are only beginning to
663 understand the ways in which genotype and the environment contribute to this process
664 and to the overall repeatability of evolution. Here, we demonstrated that the temperature
665 at which populations evolve can affect the patterns of evolution, with populations in
666 hotter environments showing greater repeatability among replicates and faster
667 convergence among genotypes. In addition, evolution at the hotter temperature results

668 in populations that are more fit in the colder temperature than vice versa. These results
669 support the growing body of work that demonstrate the importance of environment in
670 determining evolutionary trajectories of populations.

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