Synergistic Pleiotropy Overrides the Costs of Complexity in Viral Adaptation

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ABSTRACT Adaptive evolution progresses as a series of steps toward a multidimensional phenotypic optimum, and organismal or environmental complexity determines the number of phenotypic dimensions, or traits, under selection. Populations evolving in complex environments may experience costs of complexity such that improvement in one or more traits is impeded by selection on others. We compared the fitness effects of the first fixed mutations for populations of single-stranded DNA bacteriophage evolving under simple selection for growth rate to those of populations evolving under more complex selection for growth rate as well as capsid stability. We detected a cost of complexity manifested as a smaller growth rate improvement for mutations fixed under complex conditions. We found that, despite imposing a cost for growth rate improvement, strong complex selection resulted in the greatest overall fitness improvement, even for single mutations. Under weaker secondary selective pressures, tradeoffs between growth rate and stability were pervasive, but strong selection on the secondary trait resulted largely in mutations beneficial to both traits. Strength of selection therefore determined the nature of pleiotropy governing observed trait evolution, and strong positive selection forced populations to find mutations that improved multiple traits, thereby overriding costs incurred as a result of a more complex selective environment. The costs of complexity, however, remained substantial when considering the effects on a single trait in the context of selection on multiple traits.

KEYWORDS complexity; pleiotropy; bacteriophage; strong selection

ADAPTIVE evolution is characterized by the movement of a population toward a multidimensional fitness optimum determined by some number of phenotypic traits. As the number of traits under selection increases, the probability that a mutation of a given phenotypic magnitude is beneficial is hypothesized to decrease because of pleiotropy among traits (Fisher 1930). Under Fisher's geometric model (Fisher 1930), the rate of adaptation is inversely related to the number of traits under selection (Orr 2000); complex organisms cannot adapt as quickly as more simple ones and therefore incur a cost of complexity. Although these results are formulated in terms of organisms with varying numbers of phenotypic traits, they apply equivalently to organisms with the same number of phenotypic traits but with selection acting on a different number of those traits, determined by the complexity of the environment. We therefore expect adaptation to be slower under complex selective pressures and to be accomplished through mutations of smaller effects than under simpler selective conditions.

Complex environments, with multiple selection pressures acting on multiple aspects of the phenotype, often result in tradeoffs among competing functions. Predicted costs of complexity in adaptation can be manifestations of antagonistic pleiotropy. Pleiotropy is characterized by a single mutation or gene affecting more than one trait (Otto 2004; Ostman *et al.* 2011). Synergistic pleiotropy occurs when a single gene or mutation improves two or more traits (Leiby and Marx 2014). Antagonistic pleiotropy occurs when beneficial effects on a focal trait are accompanied by deleterious effects on others (Mather and Harrison 1949; Cooper and Lenski 2001; Magwire *et al.* 2010; Wenger *et al.* 2011), and such fitness tradeoffs may underlie phenomena such as senescence (Williams 1957; Hughes *et al.* 2002; Promislow 2004), cooperation (Foster *et al.* 2004), and even niche expansion by imposing constraints on the evolution of particular phenotypes (Orr 2000; Kassen 2002; MacLean *et al.* 2004;

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Remold 2012). For example, in the evolution of a specialist phenotype with a narrow niche width, mutations may fix that come with a tradeoff when exposed to an alternate environment. In the evolution of a generalist phenotype, however, mutations may fix to allow a broader niche width, but may come with a cost of reduced ability in each of the environments compared to a specialist in that environment (Levins 1968; Futuyma and Moreno 1988; Kassen 2002). As the number of phenotypic traits under selection increases, the potential for antagonistic pleiotropy also increases. In evolution experiments, the effect of a gene or mutation can be described in varying contexts, such as environments. These phenomena are often treated as pleiotropy, but they are also instances of genotype-by-environment interaction (Via and Lande 1987; Ostrowski *et al.* 2005; Paaby and Rockman 2013). Although classical approaches attempt to treat these phenomena as separate entities, microbial experiments in particular define them as pleiotropy because of the clear and distinct correlation between the selection condition imposed by the environment and the traits that are selected on. In our study, two different aspects of the environment imposed selection on two distinct traits of the bacteriophage (growth rate and capsid stability), and we identified single mutations affecting both of those traits. The nature of the traits under selection may also influence the type of pleiotropy observed, and selection on conflicting traits should increase the proportion of possible mutations that show antagonistic pleiotropy.

Proteins can exhibit antagonistic pleiotropy between stability and function; an increase in protein stability could compromise the flexibility of the protein, resulting in reduced efficiency (DePristo *et al.* 2005), although counterexamples exist (Eijsink *et al.* 2005). In viral systems, stability can have profound effects on capsid assembly kinetics because of the narrow optimal range of association energies between subunits. Weak contact energy between subunits will not allow assembly to occur, but strong contact energy can promote kinetic traps, resulting in many partially formed capsids and few free subunits available to complete assembly (Zlotnick 1994, 2003, 2005; Ceres and Zlotnick 2002). Because of the geometry of the viral capsid, a single mutation can increase the overall capsid stability, alter the association energy between capsid proteins, and disrupt proper assembly of the capsid. Other studies on lytic phages of *Escherichia coli* have suggested that tradeoffs between survival and reproduction result from changes in capsid structure (De Paepe and Taddei 2006). De Paepe and Taddei (2006) proposed that increased stability of the capsid enabled the virus to better package its DNA, but the dense packaging of DNA resulted in a slower replication rate. Selection acting on two such conflicting traits is predicted to reveal tradeoffs, where improvement of one trait comes with reduced efficiency of the other.

We characterized the first adaptive step toward a fitness optimum in a simple environment and a more complex environment to determine whether costs of complexity arise. For simple selection, we repeatedly bottlenecked populations of microvirid bacteriophages in liquid culture through random sampling until the fixation of one mutation (similarly to Rokyta *et al.* 2005). For complex selection, we employed a two-stage selection scheme to induce a two-component fitness (as described in McGee *et al.* 2014) by imposing an additional selective pressure on capsid stability between growth stages in the absence of hosts. During this second stage of the regime, we subjected populations to either extreme heat or low pH. Overall fitness was measured as a linear combination of the growth and decay rates weighted by the time spent under each condition (Handel and Bennett 2008). We varied the strength of selection by altering the time exposed to the secondary selective pressure to determine whether the starting point of the population relative to the phenotypic optimum determined whether pleiotropic mutations were observed. An additional selection pressure increases the complexity of the environment, and we predicted that increased environmental complexity would come at a cost to improvement in growth rate. Because the two traits under selection should be in conflict with each other at the molecular level, we also predicted that populations exposed to complex environments would experience tradeoffs, resulting in smaller first adaptive steps compared to those exposed to a simple selection regime.

Materials and Methods

Selection experiments

ID8 is a microvirid bacteriophage (GenBank accession no. DQ079898) characterized by a circular, single-stranded DNA genome of 5540 nucleotides encoding 11 genes with nonenveloped, tailless capsids with icosahedral geometry. ID8 was isolated by Rokyta *et al.* (2006a) and grows in laboratory conditions in *E. coli* C at 37°. Serial transfers were performed to generate beneficial growth rate mutations, as described by Rokyta *et al.* (2005). All replicate lineages were started from individual plaques isolated from a single ancestral genotype (ID8) (Rokyta *et al.* 2009) and passaged through serial transfers under a one-stage or two-stage selection regime until a single beneficial mutation fixed in the population. A culture of host cells (*E. coli* strain C) was grown to a density of $\sim 10^8$ cells/ml in 10 ml of Lysogeny Broth (10 g Tryptone, 10 g NaCl, 5 g yeast extract per liter, supplemented with 2 mM CaCl) within a 125-ml Erlenmeyer flask at 37° in an orbital shaking water bath set to 200 rpm. The hosts were prevented from evolving by being constantly replaced from our original ancestral stock. After each flask growth period, the hosts were killed. New hosts for the next growth period were derived from frozen aliquots of our host. The culture was inoculated with $\sim 10^5$ phage and allowed to propagate for 60 min, reaching a density of $\sim 10^9$–10^{10} PFU/ml. This growth phase was halted by taking an aliquot of the culture and exposing it to $CHCl_3$ to stop cell growth, followed by centrifugation to remove the cellular debris. For the one-stage selection experiments, a random sample of bacteriophage was added to

a flask of freshly grown host cells, and the process was repeated until a single mutation fixed in the population. Population sizes were monitored by plating on agar plates at two points for each growth cycle.

For the two-stage selection experiment, after the bacteriophage were grown in host cells and separated from cellular debris, they were subjected to high heat or low pH. For the high heat experiments, 1 ml of the phage-laden supernatant was separated into two 0.65-ml microcentrifuge tubes at 500 μl each. The two tubes were placed into an ice bath for 5 min to normalize the starting temperature for the subsequent heat shock. After cold exposure, the 0.65-ml tubes were transferred to hot beads in a heating block set for 80°, incubated for 5 or 12 min, and then transferred back to the ice bath for 5 min to reduce the temperature, stopping the heat shock. For the low pH conditions, 1 ml of the phage containing supernatant was transferred to a sterile glass test tube at room temperature. The pH was lowered to 1.5 with 0.5 M HCl for 3 min and brought back to pH 7 with 0.5 M NaOH. An appropriate aliquot of either the heat-shocked or the pH-shocked viruses was then transferred to the subsequent host culture and allowed to grow again. Population sizes were monitored by plating on agar plates at three points for each growth–death cycle: initial concentration prior to growth, concentration after growth, and concentration after heat shock. Population change rates were calculated on a $\log_2$ scale resulting in values of population doublings/halvings per hour.

Sequencing

We sequenced the entire genome of the final population of each lineage. Whole population sequencing allows detection of mutations that have fixed or reached high frequency. Each lineage was continued until an apparent increase in fitness was observed (~10 transfers or 30 generations). We then sequenced the last population to identify a single fixed mutation. If the population had >1 mutation, we sequenced earlier time points to determine which of the two fixed first. The correspondence between a rapid fitness increase and a single high-frequency mutation convincingly demonstrated a simple genetic basis. We then sequenced a plaque isolate from each final population per lineage to be used for all fitness assays.

Fitness assays

We measured fitness by calculating the population change rates on a $\log_2$ scale, resulting in values of population doublings/halvings per hour. Fitness was measured in conditions identical to our selective environment with isolates no more than 1 week old. We also determined fitness for mutations generated in the growth rate conditions and 5-min heat-shock conditions under the 12-min heat-shock condition. Selection for increased growth rate, γ, occurs during the growth phase of each transfer, which lasts for time τ_g. In the absence of host organisms, viral particles decay at rate δ for a time, τ_d, under harsh conditions until permissive hosts are present again. During this second stage of the regime, we subjected virus populations to either extreme heat (80°) or low pH (1.5). Fitness, ω, is measured as a combination of the growth and decay rates and the time spent under each condition, $\omega = \gamma\tau_g - \delta\tau_d$ (Handel and Bennett 2008; McGee *et al.* 2014). Fitness measures were replicated at least five times for each isolate.

Because our phage populations grow continuously rather than in discrete generations, we used Malthusian fitnesses (*i.e.*, rates) rather than more typical Wrightian fitnesses (*i.e.*, numbers of offspring). As discussed by Crow and Kimura (1970), these measures are related through a log transform. For a given Wrightian fitness w, we can convert to a Malthusian fitness $m = \log w$. Because of this relationship, the typical geometric means used to, for example, calculate mean Wrightian fitnesses across generations or environments are replaced by arithmetic means for Malthusian fitnesses.

Our fitness assays and selective conditions were identical and precluded coinfection (*i.e.*, intrahost competition) by means of a low multiplicity of infection. Our measurements therefore represent a true absolute fitness measurement for our environments, rather than the slightly less informative relative fitness measurements that would result from competition assays such as are often used in microbial systems (*e.g.*, Lenski *et al.* 1991; Lenski and Travisano 1994). In addition to being less informative, competition assays are not practical for microvirid bacteriophages because of the difficulty in introducing neutral selectable markers into their small, simple genomes to distinguish competing strains.

Statistical analyses

Pairwise comparisons and sequential Bonferroni corrections for multiple comparisons were used to determine growth rate, decay rate, and overall fitness differences from the ancestor virus.

Circle plots

Our assays and selective conditions consist of two stages: growth and decay. We estimate viral titers before and after each of these stages to estimate the growth and decay rates. The circle plots (shown in Figure 2, B–D, and Figure 3) partition these two measures and assume an optimal decay rate of zero and an optimal growth rate of 26, which is the highest value observed for this genotype or its close relatives (Rokyta *et al.* 2009). Inside the curve is beneficial overall. Inside the dotted lines is beneficial for both traits. Because our two traits are under directional rather than stabilizing selection, the plots are meaningful only in one quadrant, as shown.

The curves provided on the circle plots are not values of equal absolute fitness but of equal distance from the optimum. Consider Figure 2C. First note that the axes are scaled according to time spent in each condition as a fraction of 1 hr (*i.e.*, $\tau_g = 1$ for 1 hr of growth and $\tau_d = 0.2$ for 12 min of heat shock). The optimum is assumed to be $\gamma = 26$ and $\delta = 0$ for a total fitness of $\omega = 26 - 0 = 26$. Our wild-type ID8 showed $\tau_g\gamma = 1 \times 12.10 = 12.10$ and $\tau_d\delta = 0.2 \times 48.19 = 9.64$

Table 1 Unique mutations fixed in ID8a0 lineages under four selection regimes

Label	Selection	Times observed	Protein function	Protein name	Aa position	ΔAa	Nuc position	ΔNuc
G1	Growth	8	Spike	G	171	T→A	4493	A→G
G2	Growth	4	Spike	G	10	N→S	4011	A→G
G3	Growth	3	Spike	G	172	A→V	4496	G→A
G4	Growth	2	Spike	G	171	T→I	4494	C→T
G5	Growth	1	Pilot	H	142	G→D	4951	G→A
G6	Growth	1	Coat	F	340	A→V	3587	C→T
G7	Growth	1	Coat	F	393	I→V	3742	A→G
H5-1	5-min heat	8	Spike	G	65	T→A	4175	A→G
H5-2	5-min heat	3	Spike	G	129	T→A	4367	A→G
H5-3	5-min heat	2	Spike	G	66	N→S	4179	A→G
H5-4	5-min heat	2	Pilot	H	88	T→I	4789	C→T
H5-5	5-min heat	1	Pilot	H	43	N→D	4653	A→G
H5-6	5-min heat	1	Pilot	H	142	G→D	4951	G→A
H5-7	5-min heat	1	Pilot	H	91	G→A	4798	G→C
H5-8	5-min heat	1	Pilot	H	130	G→V	4915	G→T
H5-9	5-min heat	1	Spike	G	10	N→S	4011	A→G
H12-1	12-min heat	7	Coat	F	355	P→S	3628	C→T
H12-2	12-min heat	4	Spike	G	38	R→C	4094	C→T
H12-3	12-min heat	2	Spike	G	66	N→S	4179	A→G
H12-4	12-min heat	2	Coat	F	248	I→V	3310	G→A
H12-5	12-min heat	1	Replication	C	49	Silent	1866	C→T
H12-6	12-min heat	1	Pilot	H	71	A→T	4737	G→A
H12-7	12-min heat	1	Pilot	H	88	T→I	4789	C→T
H12-8	12-min heat	1	Pilot	H	91	G→C	4797	G→T
H12-9	12-min heat	1	Spike	G	168	R→C	4484	C→T
P1	3-min pH	8	Pilot	H	71	A→V	4738	C→T
P2	3-min pH	3	Spike	G	65	T→A	4175	A→G
P3	3-min pH	3	Pilot	H	71	A→V	4737	G→A
P4	3-min pH	2	Coat	F	77	I→T	2792	T→C
P5	3-min pH	2	Spike	G	69	S→N	4188	A→G
P6	3-min pH	1	Coat	F	393	I→V	3742	A→G
P7	3-min pH	1	Spike	G	171	T→A	4493	A→G

Positions are based on the published genome sequence of ID8 (GenBank accession no. DQ079898). Nuc, nucleotide; ΔNuc, nucleotide change; Aa, amino acid; ΔAa, amino acid change.

for a total fitness of $\omega = 12.10 - 9.64 = 2.46$. The distance of ID8 from the optimum is therefore $\Delta = \sqrt{(12-26)^2 + (9.64-0)^2} = 17.0$. The intercept for the vertical axis is therefore at $\tau_d\delta = 17$, and the intercept of the horizontal axis is therefore at $\tau_g\gamma = 26 - 17 = 9$. The points along the remainder of the curve are all equidistant from the optimum at (26, 0). For example, for a growth rate of $\tau_g\gamma = 20$, the corresponding death rate would be $\tau_d\delta = 15.9$ to maintain $\Delta = 17.0$.

Data and reagent availability

Strains are available upon request. The sequence of our ancestral strain ID8 is available in GenBank under accession no. DQ079898, and all identified mutations relative to this ancestral strain are provided in Table 1.

Results and Discussion

Parallel evolution within and between selection regimes

We identified the first fixed mutation in each of 20 replicate lineages of the single-stranded DNA (ssDNA) microvirid bacteriophage ID8 (Rokyta *et al.* 2006b) under each of four selection regimes: growth rate only at 37°, growth rate at 37° with a 5-min heat shock at 80°, growth rate at 37° with a 12-min heat shock at 80°, and growth rate at 37° with a pH shock at pH 1.5. Of the 80 lineages across the four selection regimes, we identified a total of 24 unique mutations occurring predominantly in genes encoding capsid structural proteins (F, G, and H). After each growth stage, our populations were reduced to $\sim 10^5$ phage through either random sampling (growth rate only) or selective die-off (heat or pH shock), so that our effective population sizes were modest, particularly for microbial or viral experiments. If the populations were large enough to effectively explore most mutations, each of our 20 replicate lineages under each condition would have fixed the one mutation with the largest beneficial effect as a result of clonal interference (Gerrish and Lenski 1998). The large number of unique fixed mutations showed that even simple organisms with small genome sizes can find different molecular strategies to improve fitness. Limits, however, were observed. Of the 20 replicate lineages within each selection regime, parallel molecular evolution was rampant; for each regime, single occurrences of mutations were rare (3 for growth rate only, 5 for 5-min heat shock, 5 for 12-min heat shock, and 2 for the pH shock; Table 1). The most common mutation, which was different under each type of selection, occurred eight times under growth rate conditions, eight

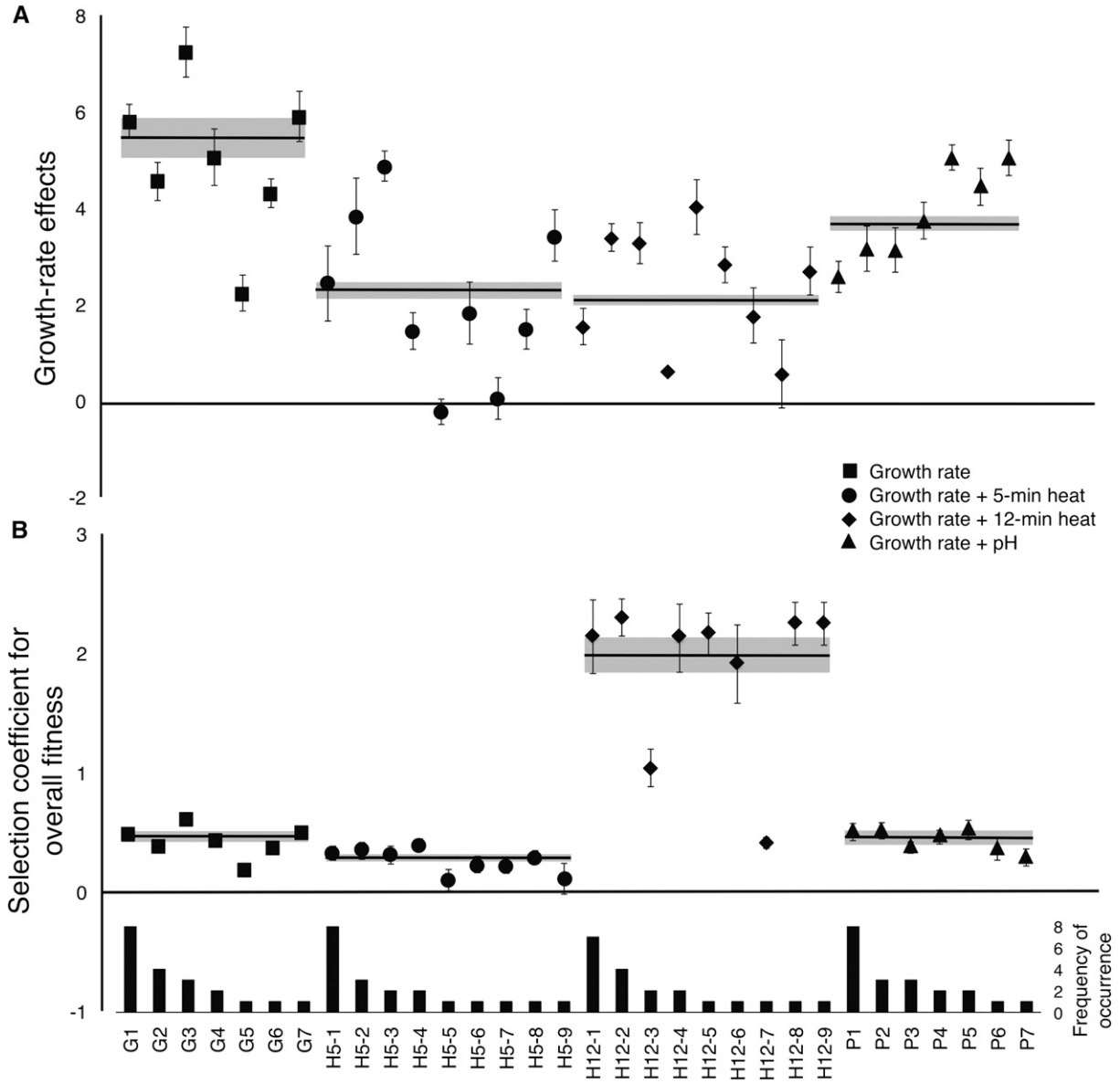

Figure 1 The strength of selection influences overall fitness effect despite a cost of complexity. (A and B) Growth rate effects (A) and selection coefficient (B) for overall fitness for growth rate (squares), 5-min heat-shock (circles), 12-min heat-shock (diamonds), and pH-shock (triangles) mutations. The solid line represents the weighted mean for each group; the shaded box represents the standard error for the weighted mean for each group. The histogram shows the frequency of occurrence for that mutation in 20 replicate lineages. Mutations generated in selection experiments with an additional selective pressure suffered a cost of reduced growth rate compared to the ancestor. Despite this cost, the selection coefficient for the overall fitness for the 12-min heat-shock mutations is higher than that for growth rate only, 5-min heat-shock, and pH-shock mutations.

times under 5-min heat-shock conditions, seven times under 12-min heat-shock conditions, and eight times under pH-shock conditions.

Although high rates of parallel evolution were detected within the same selection regime, only eight mutations were shared across different regimes, despite all lineages experiencing selection for improved growth rate. Two mutations were shared between the growth rate only and the 5-min heat-shock conditions (G2/H5-9 and G5/H5-6). By increasing the time under the heat-shock condition from 5 to 12 min, no shared mutations fixed between the growth rate only and 12-min heat-shock conditions. The longer heat-shock time increased the strength of selection on capsid stability, which may have increased the probability of fixing mutations that improved decay rate, even to the detriment of growth rate (see below). Of the 16 unique mutations that fixed under the two heat-shock conditions, only 2 fixed under both the 5-min and the 12-min heat-shock conditions (H5-3/H12-3 and H5-4/H12-3), suggesting that different sets of mutations were strongly beneficial under the different heat treatments. The pH selection regime resulted in 2 shared mutations with the growth rate only regime (G1/P7 and G7/P6), 1 shared mutation with the 5-min heat-shock regime (H5-1/P2), and 1 shared mutation with the 12-min heat-shock regime (H12-6/P3; Table 1). Parallel

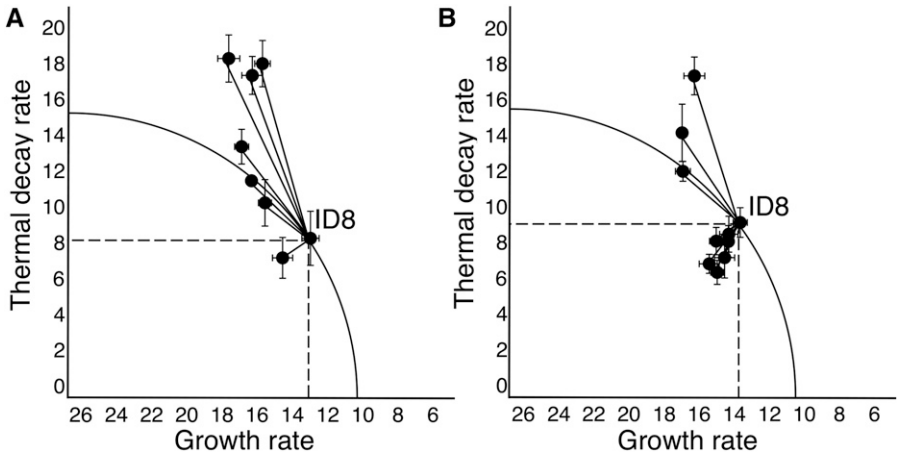

Figure 2 The type of pleiotropy depended on the strength of selection and the initial distance from the fitness optimum. (A) The starting overall fitness for ancestor ID8 under the growth rate, 5-min heat-shock, 12-min heat-shock, and pH-shock conditions. The circle plots (shown in B–D) assume an optimal decay rate of zero and an optimal growth rate of 26 (the highest value we have observed for this genotype). Inside the curve is beneficial overall. Inside the dotted lines is beneficial for both traits. (B) Five-minute mutations under 5-min heat-shock conditions; (C) 12-min mutations under 12-min heat-shock conditions; (D) pH mutations under pH-shock conditions. Under the 12-min heat-shock conditions, growth rate and decay rate were equally maladapted, resulting in selection finding more mutations synergistically pleiotropic for both traits. Under the 5-min heat-shock and pH-shock conditions, the two traits were unequally maladapted, resulting in mutations with variable pleiotropic effects (synergistic, antagonistic, or no effect).

evolution occurred at a high rate among replicate lineages within each selection regime, but not across the different treatments. Adaptation in response to each secondary selective pressure was accomplished through different sets of mutations, suggesting that each treatment imposed substantially different selective pressures on capsid stability.

Orr (2005) showed that the probability of parallel evolution can be estimated as $P = 2/(n + 1)$, given that a wild-type sequence can mutate to n different beneficial mutations. Orr's model assumes that a small number of beneficial mutations are available, that strong selection is imposed, and that the distribution of beneficial mutations conforms to that expected under extreme value theory (Orr 2005). Using Orr's equation and assuming that our observed number of mutations represents all of the beneficial mutations available (n), we calculated our expected probability of parallel evolution for each of our four selection regimes. The predicted value of P under growth rate only selection and 3-min pH-shock selection was 0.25, assuming that $n = 7$ (the number of unique mutations observed across these lineages), and $P = 0.20$ under either heat-selection regime, assuming that $n = 9$. To estimate P under each of the four selective conditions, we randomly paired all 20 replicate lineages without replacement and recorded the frequency of randomly paired lineages possessing the same mutation within each set of 10 random pairings. P was estimated for each selection regime by averaging this frequency over 1000 bootstrap data sets. Our observed estimates of the probability of parallel evolution were $\widehat{P} = 0.20$ for growth rate selection, $\widehat{P} = 0.17$ for 5-min heat selection, $\widehat{P} = 0.15$ for 12-min heat selection, and $\widehat{P} = 0.19$ for pH selection. From the bootstrap distribution, we determined the 95% confidence intervals and found that the confidence bounds were 0.0–0.40 for all four of our selection regimes. Our estimates of $\widehat{P}$, the probability of parallel evolution, observed in our experimental data agreed with estimations based on Orr's equation.

Parallel evolution is the acquisition of identical changes across independently evolving populations and has been regarded as evidence of adaptation (Stewart *et al.* 1987; Canica *et al.* 1997; Bollback and Huelsenbeck 2009), especially in short-term adaptation in response to specific selective agents (Wichman *et al.* 1999). We observed the expected high probabilities of fixation of parallel mutations predicted by Orr's equation within each selective regime, but observed surprisingly few parallel mutations across different regimes. Other studies of viral evolution have detected high rates of parallel evolution across replicate lineages (Bull *et al.* 1997; Wichman *et al.* 1999, 2000; Cuevas *et al.* 2002; Rokyta *et al.* 2005; Cuevas *et al.* 2009), and some have even identified parallel evolution across different, but related, species (Wichman *et al.* 1999; Rokyta *et al.* 2005, 2009). Our lineages exposed to differing secondary selective pressures found different adaptive solutions to solve similar problems.

Adaptation of a focal trait was impeded by selection on another one

We measured growth rate effects for populations evolved under each of our four selection regimes and found a cost of complexity for two of three sets of populations exposed to complex environmental conditions (Figure 1A). Mutations that fixed under two-stage selection for growth rate and capsid stability under heat-shock conditions had significantly lower growth rate effects compared to mutations fixed for growth rate improvement alone. Under selection for only improved growth rate, mutations had an average growth rate effect of 5.4 doublings/hr, which was significantly higher than that of the 5-min heat-shock mutations (2.5 doublings/hr; $P = 0.004$; Figure 1A) and 12-min heat-shock mutations (2.2 doublings/hr; $P = 0.002$; Figure 1A), but not significantly higher than that of the pH-shock mutations (3.3 doublings/hr; $P = 0.131$; Figure 1A; P values were Bonferroni corrected for multiple comparisons). Populations exposed to heat shock, but not pH shock, paid a significant cost of complexity, which shows that different types of selection pressures on the same trait, such as capsid stability, can have varying implications for adaptation and that costs of complexity were not universal.

Table 2 Growth rate, decay rate, and overall fitness of mutations under their selection condition

Mutation	Assay	Growth rate	Decay rate	Overall fitness
ID8	Growth rate	11.88		11.88
G1	Growth rate	17.68*		17.68*
G2	Growth rate	16.42*		16.42*
G3	Growth rate	19.11*		19.11*
G4	Growth rate	16.93*		16.93*
G5	Growth rate	14.11*		14.11*
G6	Growth rate	16.18*		16.18*
G7	Growth rate	17.77*		17.77*
ID8	5-min heat	12.12	42.38	8.59
H5-1	5-min heat	14.55*	38.31^{NS}	11.36*
H5-2	5-min heat	15.95*	53.00*	11.53*
H5-3	5-min heat	16.99*	69.17*	11.23*
H5-4	5-min heat	13.57*	18.93*	11.99*
H5-5	5-min heat	11.89^{NS}	29.63^{NS}	9.42^{NS}
H5-6	5-min heat	13.94*	40.78^{NS}	10.54*
H5-7	5-min heat	12.17^{NS}	20.32*	10.47*
H5-8	5-min heat	13.61*	30.55*	11.06*
H5-9	5-min heat	15.55*	94.67*	7.66^{NS}
ID8	12-min heat	12.10	48.19	2.46
H12-1	12-min heat	13.63**	29.61*	7.71*
H12-2	12-min heat	15.48*	36.86*	8.11*
H12-3	12-min heat	16.93*	59.60*	5.01^{NS}
H12-4	12-min heat	12.69^{NS}	25.04*	7.68*
H12-5	12-min heat	16.11*	41.71^{NS}	7.77*
H12-6	12-min heat	14.91*	38.82**	7.15*
H12-7	12-min heat	15.43**	33.71**	8.69*
H12-8	12-min heat	12.65^{NS}	23.35*	7.98*
H12-9	12-min heat	14.79*	34.03*	7.98*
ID8	3-min pH	13.30	111.25	7.74
P1	3-min pH	15.87*	85.25*	11.18*
P2	3-min pH	16.45*	95.25*	11.69*
P3	3-min pH	16.43*	116.61^{NS}	10.60*
P4	3-min pH	17.04*	115.22^{NS}	11.28*
P5	3-min pH	18.35*	132.06^{NS}	11.74*
P6	3-min pH	17.74*	144.18*	10.53*
P7	3-min pH	18.34*	167.66*	9.96**

P-values resulted from pairwise comparisons with sequential Bonferroni corrections for multiple comparisons to determine differences from the ancestor (ID8). Significance is indicated as follows: $^{*}P < 0.05$, * * $0.05 < P < 0.1$, $^{NS}P < 0.1$. Note that some mutations have significantly deleterious effects for decay rate. Growth rates are given in population doublings per hour. Decay rates are expressed as population halvings per hour.

Costs of adaptation in the evolution of niche width occur when a generalist population has reduced ability compared to a specialist population using the same resource (Levins 1968). This habitat utilization spectrum is an important dimension of the ecological niche. Specialist populations with a narrow niche range have the advantage of reduced competition and increased contact with suitable mates, but are limited to a small set of resources (Futuyma and Moreno 1988). A generalist capable of using two resources as efficiently as either specialist should be favored in a complex environment, but universally successful generalists are rarely observed (Fry 1990; Kassen 2002; Caley and Munday 2003), leading to the assumption of a cost of generalism (Levins 1968; Wilson and Yoshimura 1994; MacLean *et al.* 2004; Remold 2012). In the evolution of a generalist phenotype, multiple traits come under selection, which can result in a cost to one or more of those traits when compared to a population evolving in simple environmental conditions. Similarly, we observed reduced growth rate effects for populations subjected to a complex environment with heat shock exerting pressure for increased capsid stability. A growth rate specialist would evolve a high growth rate more quickly than a growth rate/stability generalist.

Strong selection erased the cost of complexity

The rate of fitness increase during adaptation depends on the initial fitness and the fitness effect sizes of mutations (Orr 2000). Predictions based on Fisher's geometric model show an initial rapid increase in fitness by means of mutations of larger effect followed by a gradual slowing due to mutations of small effect size as a population approaches its fitness optimum (Hartl *et al.* 1985). This pattern of diminishing returns, such that selection tends to fix mutations with progressively smaller effects as populations approach a fitness peak, is observed consistently in evolution experiments (Bull *et al.* 2000; Cuevas *et al.* 2002; Elena and Lenski 2003; Rokyta and Wichman 2009) and suggests that populations starting farther from the optimum can improve fitness more quickly than those closer to the optimum.

In our experiments, selection strength on each trait depended on the initial fitness relative to the optimum, and that optimum was consistent across conditions. For growth rate, we assume that the optimum is the highest we have ever observed for related genotypes after extensive selection for growth under our conditions (Rokyta *et al.* 2009). For decay rate, the optimum is zero. Because all ancestors shared an optimal fitness for both growth and decay rates for each selection regime, we estimated the strength of selection on the basis of the starting fitness of the ancestors. Under the 12-min heat-shock selection, the starting overall fitness of the ID8 ancestor was only 2.46 doublings/hr, whereas the ancestor under the growth rate only selection started at 11.88 doublings/hr, under pH-shock selection at 7.74 doublings/hr, and under a 5-min heat-shock selection at 8.59 doublings/hr.

We measured total fitness as a linear combination of both growth rate and decay rate (as a measure of survival) weighted by the time spent under each condition. When we compared the selection coefficients (s) for overall fitness, we determined that, despite complex environmental conditions, the selection regime with the strongest selection on the secondary trait resulted in the greatest overall fitness improvement through single mutations (Figure 1B). Mutations that fixed under the 12-min heat-shock condition resulted in a higher average fitness increase ($s = 1.97$) than mutations evolved in a simple environment (growth rate alone, $s = 0.46$, $P < 0.0001$) or in complex environments with weaker secondary selection (5-min heat shock, $s = 0.30$, $P < 0.0001$; pH shock, $s = 0.46$, $P < 0.0001$; Figure 1B). Selection was strongest for the 12-min heat-shock condition (Figure 2A) as a result of how maladapted the ancestor was to that condition compared to the other conditions, allowing for fixation of mutations of larger effect under the 12-min

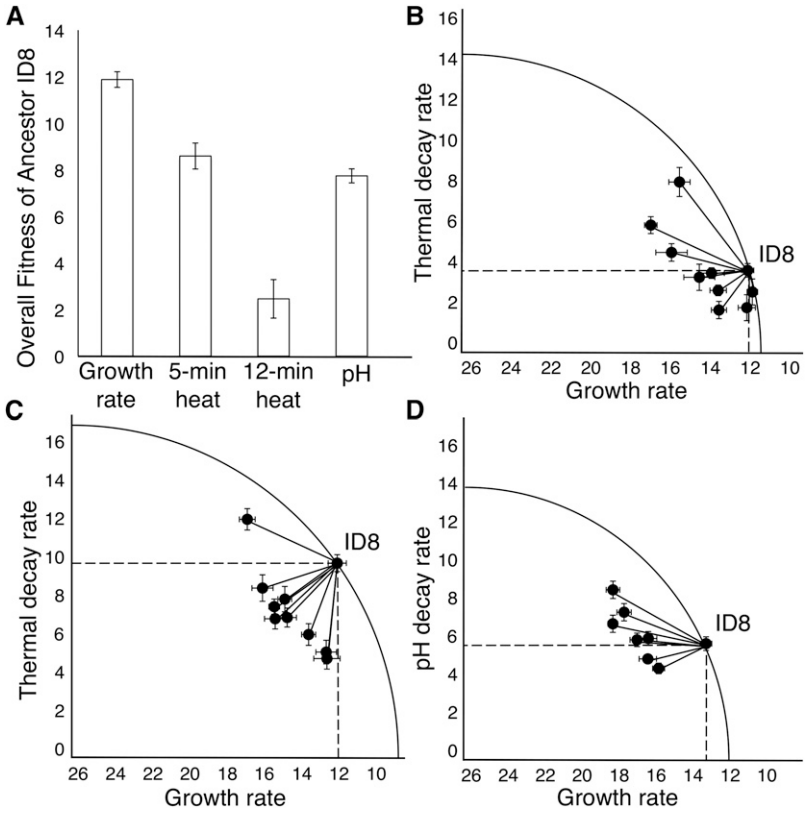

Figure 3 Antagonistic pleiotropy arose when growth rate and 5-min heat-shock mutants were subjected to a 12-min heat shock. (A) Four of the seven mutations (G1, G2, G3, and G4) that fixed in the simple selection environment for increased growth rate came with a tradeoff of significantly worsened decay rate when tested under the 12-min heat-shock condition. (B) Two mutations (H5-7 and H5-8) fixed under the 5-min heat-shock selection significantly improved decay rate under the 5-min heat shock, but we did not detect that same degree of improvement in the 12-min heat-shock condition.

heat-shock conditions (Figure 2B). Using the same genotype for our ancestors, we altered the distance from the optimum by increasing selection strength, and the effect size of fixed mutations depended on this distance. We observed a cost of reduced growth rate effects when growth rate and capsid stability were under selection, but strong selection erased that cost by fixing mutations of large effect, moving the ancestor toward the fitness optimum by greatly improving capsid stability or both growth rate and capsid stability.

In the evolution of niche width, a generalist population may begin farther from the optimum across all traits (hosts or resources), allowing it to adapt at a faster rate by fixing mutations of larger effect size. A cost of generalism may arise for one trait in the form of reduced ability compared to a specialist, but selection may improve alternate traits, resulting in improved overall fitness. Any given trait is embedded in a complex phenotype, and the effect of selection on that trait in isolation does not reveal the overall fitness of the organism in a complex environment. We identified a cost of reduced growth rate effects for populations evolving in a complex environment, but the effect of selection on a single trait, such as growth rate, does not reveal the impact of selection on overall fitness. A specialist has an advantage in adapting for a shared trait, such as growth rate, but a generalist might have an overall advantage in its rate of adaptation.

Strong selection reveals synergistic pleiotropy

Strong selection on the secondary trait resulted in the fixation of more synergistically pleiotropic mutations that improved both growth rate and capsid stability (Figure 2). For the 12-min heat-shock selection regime, five of the nine unique mutations (H12-1, H12-2, H12-6, H12-7, and H12-9) significantly improved both growth rate and capsid stability, and only one mutation (H12-3) showed antagonistic pleiotropy in the form of improved growth rate, but at a cost of worsened decay rate (H12-3; Table 2; Figure 2C). Under the 5-min heat-shock condition, only two of the nine unique mutations (H5-4 and H5-8) significantly improved both traits, with three showing tradeoffs (H5-2, H5-3, and H5-9; Figure 2B). Two of the seven pH-shock mutations (P1 and P2) significantly improved both traits, and two mutations (P6 and P7) showed antagonistic pleiotropy (Figure 2A, Figure 3B, and Table 2). Because weaker selection was imposed on the secondary trait under the 5-min heat-shock and pH-shock conditions, mutations were more likely to fix that improved growth rate; selection improved the more maladapted trait with variable impacts on the secondary trait (synergistic, antagonistic, or no pleiotropy; Figure 2). When the secondary trait was under stronger selection pressures, selection fixed more single mutations that improved fitness for both traits (Figure 2). The type of pleiotropy observed depended on the starting point relative to the fitness optimum, and this starting point was determined by selection strength.

Antagonistically pleiotropic mutations observed under complex environmental conditions have been found in experiments in which a population adapts to one condition and then its ability to function in another condition is determined. In these experiments, tradeoffs are detected because beneficial

alleles found under the initial conditions are deleterious in the alternate condition (Bull *et al.* 2000; Cooper and Lenski 2000, 2001; Lee *et al.* 2011). Our study differs because our selective regime alternated rapidly between selection for growth rate and that for decay rate, thereby selecting on multiple fitness components simultaneously. When we challenged mutants found under growth rate only and 5-min heat-shock conditions in the 12-min heat-shock condition, we observed the expected tradeoffs. Four of the seven mutations (G1, G2, G3, and G4) that fixed in the simple selection environment for increased growth rate came with a tradeoff of worsened decay rate when tested under the 12-min heat-shock condition. None of the seven growth rate mutations improved decay rate relative to the ancestor (Figure 3, Table 3). The simple selection environment allowed the fixation of mutations that improved growth rate, but came with a worsened decay rate due to the fact that capsid stability was never under selection. Three mutations (H5-4, H5-7, and H5-8) fixed under the 5-min heat-shock selection improved decay rate under the 5-min heat shock, but we did not detect that same degree of improvement in the 12-min heat-shock condition. Mutations fixed under the growth rate only or the 5-min heat-shock conditions did not significantly improve decay rate relative to the ancestor under the 12-min heat-shock conditions because selection was never imposed on the capsid to withstand 80° for an extended period of time. These results are consistent with other studies showing that improved fitness as a result of selection on one trait does not translate to improved fitness for traits not directly under selection (Bull *et al.* 2000; Cooper and Lenski 2000, 2001; Lee *et al.* 2011), but some counterexamples exist (Bennett and Lenski 2007).

We found that individual mutations gained under rapidly fluctuating selective pressures could improve both growth rate and capsid stability. When we compared the effects of beneficial mutations gained in response to selection in one condition in an alternate condition, we found the expected tradeoffs on capsid stability. When both traits are simultaneously under strong selection, we observed synergistically pleiotropic mutations that improved both traits, resulting in a larger first adaptive step toward the optimal fitness. Similar studies using bacteriophages have detected pleiotropic tradeoffs between growth rate and capsid stability, which would be expected from the conflicting nature of the viral life cycles and the stability–function tradeoff during assembly. Studies exposing bacteriophages φX174 and ID11, similar genotypes to our ID8 ancestor, to moderate temperatures (37° and 41.5°) in *E. coli* hosts resulted in changes in viral capsid proteins that were likely stabilizing and came with a cost of growth rate (Bull *et al.* 2000; Lee *et al.* 2011). One study found a single antagonistically pleiotropic mutation in bacteriophage φ6 that resulted in a tradeoff between growth rate and capsid stability after exposing evolving populations to a heat-shock selection (Dessau *et al.* 2012). Our results differ from these studies because our heat-shock temperature may have exerted a stronger selection pressure on capsid stability (80° *vs.* 40°–50°), and our two-stage selective regime fluctuated rapidly between selection for growth rate and that for decay rate, thereby selecting on multiple fitness components simultaneously. When strong selection is exerted on multiple fitness components simultaneously, selection can apparently fix single mutations that improve potentially conflicting traits, resulting in a large fitness improvement despite a cost of reduced growth rate effects.

Table 3 Growth rate, decay rate, and overall fitness of mutations across other conditions

Mutation	Assay	Growth rate	Decay rate	Overall fitness
ID8	12-min heat	12.97	40.89	4.80
G1	12-min heat	16.88*	66.44*	3.59^{NS}
G2	12-min heat	16.28*	86.31*	−0.98*
G3	12-min heat	17.61*	91.01*	−0.59*
G4	12-min heat	15.70*	89.59*	−2.22*
G5	12-min heat	14.56*	35.44^{NS}	7.47^{NS}
G6	12-min heat	15.56*	50.78^{NS}	5.40^{NS}
G7	12-min heat	16.32*	56.93^{NS}	4.94^{NS}
ID8	12-min heat	13.66	45.28	4.61
H5-1	12-min heat	15.03*	40.07^{NS}	7.02**
H5-2	12-min heat	16.98*	70.37**	2.90^{NS}
H5-3	12-min heat	16.93*	59.60*	5.01^{NS}
H5-4	12-min heat	15.43*	33.71**	8.69**
H5-5	12-min heat	14.32^{NS}	42.01^{NS}	5.91^{NS}
H5-6	12-min heat	14.56*	35.44^{NS}	7.47^{NS}
H5-7	12-min heat	14.34^{NS}	39.98^{NS}	6.34^{NS}
H5-8	12-min heat	14.98^{NS}	31.32**	8.71*
H5-9	12-min heat	16.28*	86.31*	−0.98*

P-values resulted from pairwise comparisons with sequential Bonferroni corrections for multiple comparisons to determine differences from the ancestor (ID8). Significance is indicated as follows: $^{*}P < 0.05$, $^{**}0.05 < P < 0.1$, $^{NS}P < 0.1$. Growth rates are given in population doublings per hour. Decay rates are expressed as population halvings per hour.

Fisher's geometric model assumes that any particular mutation could potentially affect all traits (*i.e.*, universal pleiotropy), but certainly does not require every mutation to affect all traits. Nonetheless, as the number of traits increases, the probability that a random mutation under this model will increase fitness decreases, as does the expected fitness gain of those that do increase fitness (Orr 2000). Wagner *et al.* (2008) showed that quantitative trait loci (QTL) in mice generally affect only a small proportion of traits and that effect sizes increase with the number of traits affected, both of which diminish the predicted costs of complexity under Fisher's geometric model. We further showed that, even when mutations affect all traits under study, sufficient mutations benefiting all traits exist to override the cost of complexity predicted under Fisher's geometric model.

Evolution in a simple environmental condition (*i.e.*, exposure to a single resource or host) results in fixation of mutations that improve the ability in that condition, but oftentimes comes with a tradeoff in the form of reduced ability when exposed to an alternate condition, resulting in a specialist phenotype (Levins 1968; Wilson and Yoshimura 1994; Kassen 2002; MacLean *et al.* 2004; Remold 2012). Generalists are more likely to evolve when the environment fluctuates, but the rate of fluctuation can influence the type of generalist that evolves (Kassen 2002; Buckling *et al.* 2007).

Fluctuations that occur too slowly relative to the organism's lifetime can result in the evolution of sequential specialists, where any adaptation gained in the previous selective environments is eroded through time if tradeoffs exist between environments (Kassen 2002). When a population fluctuates rapidly between two environments or resources, mutations fix that improve ability in both, but only if selective pressures are strong enough on both traits. Variability seen across studies on the evolution of niche breadth or host range may result from different degrees of selection strength. Our results suggest that when strong selection acts on multiple traits simultaneously, selection can find mutations that improve all without tradeoffs. While the improvement may not be equal to that of a specialist exposed to a single condition, a generalist may adapt more quickly because mutations of large effect can fix because it begins farther from the optimum for any given trait, allowing its total fitness gain to surpass that of a specialist phenotype.

Conclusions

Costs of complexity may arise in populations evolving in complex environments where improvement in one or more traits is impeded by selection on others. In a complex environment, we found that the improvement of a single trait is smaller compared to when that trait alone is under selection. But, by altering the strength of selection, single mutations fixed that improved multiple traits, allowing the total fitness increase to surpass that of a population evolving in simple environmental conditions. The costs of complexity, however, remained substantial when considering the effects on a single trait in the context of selection on multiple traits. Evolution in a complex environment must be viewed in the context of multiple selective pressures acting on multiple, interacting traits that make up a phenotype. Although we detected a cost of complexity for a single trait, by analyzing the network of traits that are the targets of selection and the interactions between them, we determined that populations in complex selection conditions can overcome such a cost by fixing mutations exhibiting synergistic pleiotropy.

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Literature Cited

Bennett, A., and R. Lenski, 2007 An experimental test of evolutionary trade-offs during temperature adaptation. Proc. Natl. Acad. Sci. USA 104: 8649–8654.

Bollback, J. P., and J. P. Huelsenbeck, 2009 Parallel genetic evolution within and between bacteriophage species of varying degrees of divergence. Genetics 181: 225–234.

Buckling, A., M. Brockhurst, M. Travisano, and P. Rainey, 2007 Experimental adaptation to high and low quality environments under different scales of temporal variation. J. Evol. Biol. 20: 296–300.

Bull, J. J., M. R. Badgett, H. A. Wichman, J. P. Huelsenbeck, D. M. Hillis *et al.*, 1997 Exceptional convergent evolution in a virus. Genetics 147: 1497–1507.

Bull, J. J., M. R. Badgett, and H. A. Wichman, 2000 Big-benefit mutations in a bacteriophage inhibited with heat. Mol. Biol. Evol. 17: 942–950.

Caley, M., and P. L. Munday, 2003 Growth trades off with habitat specialization. Proc. R. Soc. Lond. B Biol. Sci. 270: S175–S177.

Canica, M. M., C. Y. Lu, R. Krishnamoorthy, and G. C. Paul, 1997 Molecular diversity and evolution of bla genes encoding beta-lactamases resistant to clavulanic acid in clinical *E. coli*. J. Mol. Evol. 44: 57–65.

Ceres, P., and A. Zlotnick, 2002 Weak protein-protein interactions are sufficient to drive assembly of hepatitis B virus capsids. Biochemistry 41: 11525–11531.

Cooper, V. S., and R. E. Lenski, 2000 The population genetics of ecological specialization in evolving *Escherichia coli* populations. Nature 407: 736–739.

Cooper, V. S., and R. E. Lenski, 2001 Evolution of thermal dependence of growth rate of *Escherichia coli* populations during 20,000 generations in a constant environment populations. Evolution 55: 889–896.

Crow, J. F., and M. Kimura, 1970 *An Introduction to Population Genetics Theory*. Harper & Row, New York/Evanston, IL/London.

Cuevas, J. M., S. F. Elena, and A. Moya, 2002 Molecular basis of adaptive convergence in experimental populations of RNA viruses. Genetics 162: 533–542.

Cuevas, J. M., S. Duffy, and R. Sanjuán, 2009 Point mutation rate of bacteriophage φx 174. Genetics 183: 747–749.

De Paepe, M., and F. Taddei, 2006 Viruses' life history: towards a mechanistic basis of a trade-off between survival and reproduction among phages. PLoS Biol. 4: e193.

DePristo, M. A., D. M. Weinreich, and D. L. Hartl, 2005 Missense meanderings in sequence space: a biophysical view of protein evolution. Nat. Rev. Genet. 6: 678–687.

Dessau, M., D. Goldhill, R. McBride, P. Turner, and Y. Modis, 2012 Selective pressure causes an RNA virus to trade reproductive fitness for increased structural and thermal stability of a viral enzyme. PLoS Genet. 8: e1003102.

Eijsink, V. G. H., S. Gåseidnes, T. V. Borchert, and B. van den Burg, 2005 Directed evolution of enzyme stability. Biomol. Eng. 22: 21–30.

Elena, S. F., and R. E. Lenski, 2003 Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. Nat. Rev. Genet. 4: 457–469.

Fisher, R. A., 1930 *The Genetical Theory of Natural Selection*. Oxford University Press, Oxford.

Foster, K. R., G. Shaulsky, J. E. Strassmann, D. C. Queller, and C. R. L. Thompson, 2004 Pleiotropy as a mechanism to stabilize cooperation. Nature 431: 693–696.

Fry, J., 1990 The evolution of ecological specialization. Am. Nat. 136: 569–580.

Futuyma, D., and G. Moreno, 1988 Trade-offs in fitness on different hosts: evidence from a selection experiment with a phytophagous mite. Annu. Rev. Ecol. Syst. 19: 207–233.

Gerrish, P. J., and R. E. Lenski, 1998 The fate of competing beneficial mutations in an asexual population. Genetica 102/103: 127–144.

Handel, A., and M. R. Bennett, 2008 Surviving the bottleneck: transmission mutants and the evolution of microbial populations. Genetics 180: 2193–2200.

Hartl, D. L., D. E. Dykhuizen, and A. M. Dean, 1985 Limits of adaptation: the evolution of selective neutrality. Genetics 111: 655–674.

Hughes, K., J. Alipaz, J. Drnevich, and R. Reynolds, 2002 A test of evolutionary theories of aging. Proc. Natl. Acad. Sci. USA 99: 14286–14291.

Kassen, R., 2002 The experimental evolution of specialists, generalists, and the maintenance of diversity. J. Evol. Biol. 15: 173–190.

Lee, K. H., C. R. Miller, A. C. Nagel, H. A. Wichman, P. Joyce *et al.*, 2011 First-step mutations for adaptation at elevated temperature increase capsid stability in a virus. PLoS One 6: e25640.

Leiby, N., and C. Marx, 2014 Metabolic erosion primarily through mutation accumulation, and not tradeoffs, drives limited evolution of substrate specificity in Escherichia coli. PLoS Biol. 12: 1–10.

Lenski, R. E., and M. Travisano, 1994 Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. Proc. Natl. Acad. Sci. USA 91: 6808–6814.

Lenski, R. E., M. R. Rose, S. C. Simpson, and S. C. Tadler, 1991 Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. Am. Nat. 138: 1315–1341.

Levins, R., 1968 *Evolution in Changing Environments*. Princeton University Press, Princeton, NJ.

MacLean, R. C., G. Bell, and P. B. Rainey, 2004 The evolution of a pleiotropic fitness tradeoff in *Pseudomonas fluorescens*. Proc. Natl. Acad. Sci. USA 101: 8072–8077.

Magwire, M., A. Yamamoto, M. Carbone, N. Roshina, A. Symonenko *et al.*, 2010 Quantitative and molecular genetic analyses of mutations increasing Drosophila life span. PLoS Genet. 6: e1001037.

Mather, K., and B. Harrison, 1949 The manifold effect of selection. Heredity 23: 131–162.

McGee, L., E. Aitchison, S. Caudle, A. Morrison, L. Zheng *et al.*, 2014 Payoffs, not tradeoffs, in the adaptation of a virus to ostensibly conflicting selective pressures. PLoS Genet. 10: e1004611.

Orr, H. A., 2000 Adaptation and the cost of complexity. Evolution 54: 13–20.

Orr, H. A., 2005 The probability of parallel evolution. Evolution 59: 216–220.

Ostman, B., A. Hintze, and C. Adami, 2011 Impact of epistasis and pleiotropy on evolutionary adaptation. Proc. R. Soc. Lond. B Biol. Sci. 279: 247–256.

Ostrowski, E., D. Rozen, and R. Lenski, 2005 Pleiotropic effects of beneficial mutations in *Escherichia coli*. Evolution 59: 2343–2352.

Otto, S. P., 2004 Two steps forward, one step back: the pleiotropic effects of favoured alleles. Proc. R. Soc. Lond. B Biol. Sci. 271: 705–714.

Paaby, A., and M. Rockman, 2013 The many faces of pleiotropy. Trends Genet. 2: 147–156.

Promislow, D. E. L., 2004 Protein networks, pleiotropy and the evolution of senescence. Proc. R. Soc. Lond. B Biol. Sci. 271: 1225–1234.

Remold, S. K., 2012 Understanding specialism when the jack of all trades can be the master of all. Proc. Natl. Acad. Sci. USA 98: 11388–11393.

Rokyta, D. R., and H. A. Wichman, 2009 Genic incompatibilities in two hybrid bacteriophages. Mol. Biol. Evol. 26: 2831–2839.

Rokyta, D. R., P. Joyce, S. B. Caudle, and H. A. Wichman, 2005 An empirical test of the mutational landscape model of adaptation using a single-stranded DNA virus. Nat. Genet. 37: 441–444.

Rokyta, D. R., C. J. Beisel, and P. Joyce, 2006a Properties of adaptive walks on uncorrelated landscapes under strong selection and weak mutation. J. Theor. Biol. 243: 114–120.

Rokyta, D. R., C. L. Burch, S. B. Caudle, and H. A. Wichman, 2006b Horizontal gene transfer and the evolution of microvirid coliphage genomes. J. Bacteriol. 188: 1134–1142.

Rokyta, D. R., Z. Abdo, and H. A. Wichman, 2009 The genetics of adaptation for eight microvirid bacteriophages. J. Mol. Evol. 69: 229–239.

Stewart, C.-B., J. Schilling, and A. Wilson, 1987 Adaptive evolution in the stomach lysozymes of foregut fermenters. Nature 330: 401–404.

Via, S., and R. Lande, 1987 Evolution of genetic-variability in a spatially heterogeneous environment – effects of genotype-environment interaction. Genet. Res. 49: 147–156.

Wagner, G. P., J. P. Kenney-Hunt, M. Pavlicev, J. R. Peck, D. Waxman *et al.*, 2008 Pleiotropic scaling of gene effects and the 'cost of complexity'. Nature 452: 470–473.

Wenger, J., J. Piotrowski, S. Nagarajan, K. Chiotti, G. Sherlock *et al.*, 2011 Hunger artists: yeast adapted to carbon limitation show trade-offs under carbon sufficiency. PLoS Genet. 7: e1002202.

Wichman, H. A., M. R. Badgett, L. A. Scott, C. M. Boulianne, and J. J. Bull, 1999 Different trajectories of parallel evolution during viral adaptation. Science 285: 422–424.

Wichman, H. A., L. A. Scott, C. D. Yarber, and J. J. Bull, 2000 Experimental evolution recapitulates natural evolution. Philos. Trans. R. Soc. Lond. B Biol. Sci. 355: 1677–1684.

Williams, G. C., 1957 Pleiotropy, natural selection, and the evolution of senescence. Evolution 11: 398–411.

Wilson, D., and J. Yoshimura, 1994 On the coexistence of specialists and generalists. Am. Nat. 144: 692–707.

Zlotnick, A., 1994 To build a virus capsid: an equilibrium model of the self assembly of polyhedral protein complexes. J. Mol. Biol. 241: 59–67.

Zlotnick, A., 2003 Are weak protein-protein interactions the general rule in capsid assembly? Virology 315: 269–274.

Zlotnick, A., 2005 Theoretical aspects of virus capsid assembly. J. Mol. Recognit. 18: 479–490.

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