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Delayed transmission selects for increased survival of vesicular stomatitis virus

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Life-history theory predicts that traits for survival and reproduction cannot be simultaneously maximized in evolving populations. For this reason, in obligate parasites such as infectious viruses, selection for improved between-host survival during transmission may lead to evolution of decreased within-host reproduction. We tested this idea using experimental evolution of RNA virus populations, passaged under differing transmission times in the laboratory. A single ancestral genotype of vesicular stomatitis virus (VSV), a negative-sense RNA Rhabdovirus, was used to found multiple virus lineages evolved in either ordinary 24-h cell-culture passage, or in delayed passages of 48 h. After 30 passages (120 generations of viral evolution), we observed that delayed transmission selected for improved extracellular survival, which traded-off with lowered viral fecundity (slower exponential population growth and smaller mean plaque size). To further examine the confirmed evolutionary trade-off, we obtained consensus whole-genome sequences of evolved virus populations, to infer phenotype–genotype associations. Results implied that increased virus survival did not occur via convergence; rather, improved virion stability was gained via independent mutations in various VSV structural proteins. Our study suggests that RNA viruses can evolve different molecular solutions for enhanced survival despite their limited genetic architecture, but suffer generalized reproductive trade-offs that limit overall fitness gains.

KEY WORDS: **Adaptation, evolutionary genomics, experimental evolution, life-history evolution, trade-offs, virus.**

Ecological circumstances will dictate how parasites evolve to maximize their fitness, which is the sum effect of all parasite traits contributing to within-host reproduction and between-host transmission. Life-history theory suggests that traits for reproduction and survival cannot always be simultaneously maximized in evolving populations (Stearns 1989); for parasites, this means that evolutionary improvements in within-host reproduction should lead to concomitant reductions in extra-host survival (and vice versa). Thus, assuming that pleiotropy often leads to such trade-offs, selection for a mutation that improves within-host growth by a parasite may cause the parasite to suffer lowered survival as it transmits between hosts, and vice versa. Such conflicting selection pressures can lead to evolved compromises between parasite traits responsible for reproduction and survival. For this reason, life-history theory would generally predict that parasites with enhanced survival should suffer reduced reproduction. In addition, theory on the evolution of parasite virulence has sometimes combined extra-host survival and within-host reproduction of parasites into the same mathematical model, to examine how changes in parasite survival should relate to evolved virulence (parasite induced damage to the host) (e.g., Bonhoeffer et al. 1996; Gandon 1998; Kamo and Boots 2004). One possibility is that parasites with extreme extra-host stability can experience a decreased cost

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of an extreme exploitation strategy, because a parasite that can survive outside its host will enhance its chances of being transmitted; intuitively, this suggests that high survival of free-living stages should coincide with increased parasite virulence (Ewald 1987, 1993, 1994). Some mathematical models support the idea that parasite virulence should increase with survival, particularly when the host–parasite system is in disequilibrium (Bonhoeffer et al. 1996), when multiple infection (and hence, intrahost competition) is common (Gandon 1998), or when virulence evolves independently of transmission (Kamo and Boots 2004); however, these same models identify ecological conditions where greater parasite stability should coincide with lower parasite virulence, echoing the predictions from life-history theory. Caraco and Wang (2008) combine theories of life-history evolution and parasite virulence while considering functional dependencies between parasite traits, to predict that anatomical and physiological adaptations that enhance parasite survival should often trade-off with efficient intrahost reproduction. Thus, the existing theory offers mixed support for the notion that evolutionary increases in parasite survival should coincide with greater virulence. However, few empirical studies have manipulated ecological conditions to examine the interplay between selection for parasite survival versus that for reproduction, and whether constraints prevent these traits from being simultaneously maximized (e.g., Olmstead et al. 1984; Ebert and Mangin 1997; Elena 2001; Cooper et al. 2002).

Infectious viruses are nonmetabolizing microbes, which do not experience the metabolic trade-offs sometimes suffered by cellular organisms. But it is evident that viruses experience life-history compromises analogous to those observed in evolving populations of cellular life forms (e.g., De Paepe and Taddei 2006; Ogbunugafor et al. 2013). The proteins and hereditary nucleic acids (RNA or DNA) of infectious virus particles may be easily degraded by extra-host environmental stressors, such as extremes in temperature and pH. These stressors reduce the ability for virions to successfully infect target host cells or tissues. One comparative study showed that increased survival (virion stability) was correlated with reduced reproduction, across a collection of lytic bacteriophages that infect *Escherichia coli* bacteria (De Paepe and Taddei 2006). A general mechanistic explanation could be that denser packed viral nucleic acid allows greater stability while slowing the rate of phage genome replication (De Paepe and Taddei 2006). However, it remains unclear which ecological circumstances best explain the particular reproduction/survival compromise for each phage type in this comparative analysis, whereas these details may be revealed through experiments that attempt to directly select for increased virus survival and which measure the subsequent effects on reproduction.

Two prior empirical studies involved experimental evolution of vesicular stomatitis virus (VSV), and yielded results concerning evolved trade-offs between virus survival and reproduction (Elena 2001; Ogbunugafor et al. 2013). VSV is a zoonotic *Vesiculovirus* in the family *Rhabdoviridae* that is vector-transmitted to mammal hosts via biting insects or by direct contact, and which provides a useful biological model for studying evolution of RNA viruses in general, and arthropod-borne viruses (arboviruses) in particular (Holland et al. 1991; Turner and Elena 2000; Elena et al. 2001; Remold et al. 2008; Turner et al. 2010). VSV has an ~11 kb negative-sense ssRNA genome, encoding five proteins: the nucleocapsid (N) protein that tightly encapsidates the genomic RNA, phosphoprotein (P) and large (L) protein that make up the polymerase, glycoprotein (G) involved in cell-surface binding and infection initiation via membrane fusion, and matrix (M) protein important for virion formation and inhibition of host antiviral gene expression (Rose and Whitt 2001). Elena (2001) manipulated the timing of transmission (i.e., serial passage at 12, 24, and 48 h) in experimentally evolved VSV populations, and observed that rapidly transmitted viruses evolved higher virulence (increased fecundity per infection), whereas slowly transmitted viruses evolved lower virulence. Furthermore, the study measured growth curves for the evolved VSV populations during 56 h after initial infection, to infer that viruses evolved under delayed (48 h) transmission presented decreased decay rates (enhanced survival) and lowered reproduction (reduced virulence). Although this outcome was consistent with the survival-reproduction trade-off predicted by some theory, the study did not examine possible mechanisms underlying apparent trade-offs (e.g., using phenotype–genotype associations), and indirectly inferred virus survival from fitting logistic curves to population growth data rather than measuring survival directly. A later study by Ogbunugafor et al. (2013) examined experimental evolution of VSV populations on cancer-derived and/or noncancerous cells (see also Turner and Elena 2000; Remold et al. 2008; Turner et al. 2010). The study showed that selection strictly on cancer cells led to increased extracellular survival of VSV populations, attributed to relatively faster cell-to-cell spread of viruses on cancer cells and the more rapid senescence of these cell types, either of which may select for enhanced virus stability between host-cell replenishment. However, Ogbunugafor et al. (2013) showed only weak evidence that increased virion survival coincided with decreased intrahost reproduction, and this outcome was confounded by cell type (i.e., it was not observed across all host treatments), preventing the possibility of drawing general conclusions on evolved survival/reproduction trade-offs in VSV.

Here, we sought to examine the evolution of survival/reproduction trade-offs in VSV, by holding the host-type constant while solely manipulating the transmission time experienced by viruses between infections. In particular, we allowed all VSV lineages to be experimentally evolved for 30 passages (120 viral generations) on their typical laboratory host (baby hamster kidney [BHK] cells), in replicated passage regimes under

ordinary conditions (24-h passage) versus delayed transmission (48-h passage). Moreover, to further efforts at exploring correlated evolution in RNA viruses such as VSV (e.g., Alto and Turner 2010; Turner et al. 2010; Alto et al. 2013), we capitalized on the populations evolved in the current study, to determine whether changes in virus survival in the selected environment (37°C), coincided with increased or decreased survival at the unselected thermal extremes for VSV growth on BHK cells: 14°C and 40°C (Alto and Turner 2010). Data on correlated fitness improvements in alternate temperatures are particularly useful for arboviruses, because these pathogens are poised to expand into environments at the extremes of their temperature niche, alongside vectors such as mosquitoes that are currently undergoing geographic range expansion (Alto and Juliano 2001). Although similar to the experiment by Elena (2001), our study differed because we directly measured extracellular survival (ECS) as a virus trait in selected and unselected environments, gauged survival up to 96 h after initial infection, and conducted whole-genome sequencing to explore molecular changes underlying phenotypic evolution in VSV populations. This approach allowed us to test (1) if delayed transmission selects for increased ECS of VSV virions, including thermal extremes, (2) if reproduction (fecundity) is maximized under minimal transmission time, (3) and whether there exists a trade-off between ECS and fecundity for VSV.

Methods

VIRUSES AND CULTURE CONDITIONS

All viruses were derived from a clonal isolate of a low-passage, laboratory-adapted strain of Mudd Summer VSV Indiana serotype (hereafter referred to as wild type). Sequencing (see below) of this wild-type clone (Genbank # KF880907) showed six allele substitutions that distinguished it from the published Mudd-Summers strain sequence (Genbank #EU849003.1). Viruses and host cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) with 10% heat-inactivated fetal bovine serum and antibiotics penicillin and streptomycin. Hosts for infection were BHK cells grown in 25 cm^2 tissue-culture flasks at 37°C, 95% relative humidity, and 5% CO_2 atmosphere to achieve confluent monolayers of $\sim 10^5$ cells/cm^2. Virus particles were enumerated by plaque assays using confluent BHK cell monolayers with DMEM-0.7% low-melt agarose overlays.

EXPERIMENTAL EVOLUTION OF VIRAL POPULATIONS

A single clone of VSV wild type was used to find four replicate populations in each of two experimental treatments: ordinary transmission (24 h) and delayed transmission (48 h), where time refers to the hours elapsed between sequential passage on freshly grown (24 h old) BHK host monolayers. Consistent with prior studies in VSV (e.g., Elena 2001; Alto et al. 2013), preliminary assays ($n = 5$) showed that wild-type VSV reliably completed its growth on BHK cells by 24 h postinfection (hpi) at 37°C to achieve a maximum mean titer of 10.07 ($\pm$ 0.08 SD) $\log_{10}$ plaque-forming units (pfu) per mL. The mean titer at 48 h was somewhat lower, 9.59 ($\pm$ 0.28 SD) $\log_{10}$ pfu/mL, which is consistent with earlier VSV studies (Elena 2001) and the mean titers at these time points significantly differed (t-test with $t = 3.63$, df $= 8$, $P = 0.007$), owing to the expected decay rate of the VSV population in the 12 h after growth has ceased. Furthermore, throughout the study we visualized infected monolayers via light microscopy, and observed that the infected BHK host-cell monolayers were completely destroyed within 24 h, with no obvious population differences within and among treatments. Thus, these observations confirmed that VSV growth was completed within 24 h, indicating that the generations of VSV growth per passage were equal across the two treatments, but that ordinary-transmission viruses experienced minimal extracellular exposure whereas delayed-transmission virus populations experienced $\sim$24 h of extracellular exposure. The propagation passage cycle was repeated by harvesting virus at 24 hpi (ordinary transmission) or 48 hpi (delayed transmission) and using a 10^{-4} dilution of each virus population to infect a new BHK monolayer. We note that this design ensured cell monolayers were infected at a multiplicity of infection of roughly 0.01 viruses per cell to avoid the appearance of defective-interfering particles characteristic of high multiplicity infections with VSV (Horodyski et al. 1983). The experimental evolution proceeded for 30 passages, or 120 virus generations (four generations per passage). Samples from each population were stored at intervals of five passage cycles at -80°C for further investigation.

EXTRACELLULAR SURVIVAL

We amplified a test virus under its relevant treatment conditions described above (24 or 48 hpi), and harvested the supernatant to obtain a cell-free lysate. We then diluted the lysate for each test virus to achieve $\sim 10^6$ pfu/mL of virus in 10 mL of cell-free DMEM, placed in a 25 cm^2 culture flask. We prepared a total of 15 flasks for each test virus, allowing threefold replication of an assay that monitored survival (remaining titer) of the virus by destructively sampling flasks at five time points (6, 12, 24, 36, and 48 h), in assays performed at three incubation temperatures (14°C, 37°C, and 40°C). We used plaque assays to estimate the remaining virus density (titer; $\log_{10}$ pfu/mL) at each of the five postincubation time points.

FECUNDITY MEASUREMENTS

We examined virus fecundity via 6-h growth productivity assays of virus populations; we chose this duration to minimize the effects on productivity of nonfecundity traits (e.g., survivorship), which become more salient with longer incubation times. Roughly

10^4 viruses of each experimental lineage were allowed to infect replicate 25 cm^2 flasks (three replicates per lineage) containing confluent BHK host cells under a multiplicity of infection (ratio pfu to cells) of 0.01. The viruses were enumerated using plaque assays and these data were then $\log_{10}$ transformed. Delta-$\log_{10}$ titer was calculated as the 6-h enumerated titer divided by the population initial titer (10^4 pfu).

As a second proxy for fecundity, we examined plaque diameter. Similar to reproduction rate, fecundity is a measure of the resulting viral progeny that arise from a single instance of a viral infection. Plaque size is an established measure of virus fecundity or fitness, and has been previously used in several viral systems (e.g., Burch and Chao 2000; Abedon and Culler 2007), including VSV (Sanjuan et al. 2007; Ogbunugafor et al. 2013). The diameter of the plaque is proportional to the number of cells that a virus destroys, and is therefore related to virus productivity in the given time period. Data for the fecundity experiment were gathered from randomly selected plaques from 80 confluent six-well plates of BHK grown and infected on the same day. Plates were scanned with their lids off and a light source placed underneath the scanner. Using Adobe Photoshop, each plate was magnified to 200% and its contrast increased by adding a Brightness/Contrast filter of +20 points; 200% magnification was chosen as a compromise between resolution and the proportional exaggeration of differences in plaque size. Plaque size was measured using the Ruler tool, which calculates the distance between any two points in the workspace. The tool was set to collect data in millimeters with a standard error of 0.1 mm. Twenty plaques were measured for each viral subtype.

RNA ISOLATION, REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION, AND SEQUENCING OF GENOMES

Prior to sequencing, virus strains were amplified under the relevant selective conditions (24 or 48 h), and no substantial differences in titer were observed (i.e., $\approx$10 $\log_{10}$ pfu/mL; data not shown). Genomic viral RNA was isolated from nine strains (eight evolved populations + the wild-type ancestor) using Trizol LS reagent (Invitrogen) following manufacturer protocol suggestions. Precipitated RNA was resuspended in nuclease-free H_2O. cDNA was generated by reverse transcription with Superscript II (Invitrogen) using random hexamer primers. The majority of the genome sequence was amplified via polymerase chain reaction (PCR) using seven primer pairs, generating overlapping PCR fragments 2–3 kb in length. Following gel purification, the fragments were sequenced via dye termination (Sanger) at the Yale University DNA Sequencing Facility. Coverage was generated at two- to threefold using overlapping fragments and multiple directional sequencing primers.

Our approach generates a consensus genomic sequence for each population, rather than a defined genome sequence of representative clones. The result is a "minimum set" of variable alleles in the population, because alleles at frequencies below ~0.08 are below the limit of detection using this method (Remold et al. 2008).

GENOME ASSEMBLY AND ALIGNMENT

All sequences were reviewed by eye following genome assembly using DNA Workbench 6 (CLC Bio) for Mac OS10.6.8. Where multiple quality sequencing readings resulted in strong secondary peaks at a single locus, the population was concluded to be polymorphic at that locus. Polymorphic loci identified in the population sequencing were included in the results, with the dominant (consensus) allele denoted. Consensus sequences from the evolved populations were aligned to the ancestral sequence generated in this study.

DATA MANIPULATION AND STATISTICAL ANALYSES

Datasets were analyzed by a fit least squares model performed using JMP statistical software package (version 10, SAS Institute Inc., Cary, NC). To analyze ECS, time point and transmission were treated as model effects (Table S1). For productivity and plaque size datasets, transmission alone was a model effect under the ANOVA analysis (Table S2). Means, 95% confidence intervals, and $\log_{10}$ data transformations were calculated and graphed using GraphPad Prism (version 6.00; GraphPad Software, La Jolla, CA).

Results/Discussion

EVOLVED CHANGES IN ECS OF VIRUSES

We predicted that ECS of VSV virions would be an evolvable trait in our study, because earlier work inferred that survival of VSV improved under prolonged transmission but not under ordinary transmission (Elena 2001). Also, our prior study showed that ECS could evolutionarily change when VSV was cultured strictly on cancer-derived HeLa cells (Ogbunugafor et al. 2013). Here, we hypothesized that prolonged timing-of-transmission would be a sufficient environmental variable to select for VSV variants with increased ECS, when viruses were cultured on BHK cells. We examined this idea by directly measuring ECS of VSV lineages for up to 48 h after viruses were removed from cell monolayers (i.e., up to 96 h after initial infection, which was roughly twice the duration examined by Elena 2001). These assays were performed using the four evolved lineages in each treatment group as the experimental replicates. We measured ECS at the temperature experienced by virus populations during their experimental evolution: 37°C. Also, we performed identical assays examining ECS at 14°C and 40°C, which are temperatures near the thermal

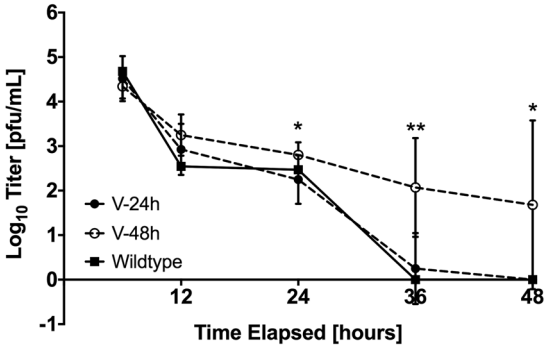

Figure 1. Mean extracellular survival through time at 37°C for VSV populations evolved under differing transmission. Delayed-transmission lineages (open circles) show significantly increased survival at 24, 36, and 48 h, relative to otherwise identical populations passaged under ordinary transmission (solid circles). Mean survival data for the wild-type genotype (solid squares) are depicted for comparison. Error bars represent 95% confidence intervals.

extremes for VSV growth (Alto and Turner 2010); measuring ECS at these extreme temperatures allowed us to examine correlated responses to selection, and would indicate whether changes in survival in the selective environment influenced VSV performance at harsher unselected temperatures.

Results observed in the selective 37°C environment (Fig. 1) showed that delayed-transmission virus populations were significantly advantaged in mean ECS after extended periods spent outside of cells (24, 36, and 48 h), relative to their counterparts evolved under ordinary-transmission times (Table S1; ANOVA with transmission effect $F_{(1,30)} = 26.69$, $P < 0.0001$). Interestingly, the delayed-transmission lineages also showed higher mean ECS values at 36 and 48 h in assays conducted at 14°C (Fig. S1A), suggesting that these populations had evolved correlated improvements in survival at this unselected temperature. However, our analysis detected only a borderline nonsignificant difference between treatment groups (Table S1; ANOVA with transmission effect $F_{(1,30)} = 3.64$, $P = 0.066$). In contrast, measurements of ECS were similar across treatment groups in assays conducted at 40°C, with no significant differences observed between groups (Fig. S1B; Table S1; ANOVA with transmission effect $F_{(1,30)} = 0.89$, $P = 0.352$). Overall, the data indicated that we correctly predicted ECS of VSV lineages should selectively improve when transmission time was delayed. However, this outcome was specific to survivorship in the selective 37°C environment, and did not lead to correlated improvement in ECS in the unselected environments near the thermal limits for VSV growth. Also, the data showed that all evolved VSV populations survived poorly at 40°C, perhaps owing to generalized detrimental effects of high temperature, such as destabilization of virus structural proteins.

Our results (Fig. 1) indicated that manipulation of transmission time alone was sufficient to select for improved VSV survival (see also Elena 2001); although we previously showed that evolution of differential survival among VSV strains was possible, host treatment was a confounding factor that prevented our ability to draw generalized conclusions (Ogbunugafor et al. 2013). In contrast, the current study showed that an abiotic effect (time spent outside of cells) could lead to divergence among RNA virus populations in the phenotypic trait, ECS. Although most virology studies strictly concern growth differences among viral genotypes in the laboratory, the natural "transmission ecology" of viruses is known to sometimes include extended periods outside of cells/hosts, such as transmission that is airborne, waterborne, or in association with fomites (inanimate objects). Our findings highlight that it may be useful to compare/contrast survival differences among virus types, especially if their historical ecology is known or suspected to involve extended durations outside of cells. These studies could better illuminate the evolutionary ecology of pathogenic RNA viruses, such as rotavirus that is classically shown to persist for long periods on inanimate surfaces (Estes et al. 1979). More generally, by experimentally determining conditions that cause ECS to change, one may pinpoint how to develop viruses with maximal or minimal virion stability, useful for therapeutic and industrial applications with viruses where short-term versus long-term virus delivery is desired.

We note an additional result observed in plaque assays that were used to measure ECS. Recall that cell-free virus samples were exposed to a challenge temperature, and the survivors were enumerated via plaque assays at the permissive 37°C temperature (see Methods). Here we found an overall effect, where plaques of viruses incubated at the three challenge temperatures differed considerably in size (results not shown). In particular, viruses that were exposed to extreme temperatures of 14°C and 40°C consistently produced smaller plaques, relative to those incubated at 37°C, regardless of their treatment group. One explanation is that viruses undergo an environmental "shock" when placed in an extreme temperature such at 14°C or 40°C, causing them to form relatively smaller plaques under the benign temperature of 37°C. This result suggests an epigenetic "memory" that is retained in the viral progeny that form a plaque initiated by a virus subjected to temperature shock; presumably, fecundity or some other key fitness component is adversely affected by the temperature shock such that this disadvantage percolates across virus generations as the plaque forms. Alternatively, the temperature-shocked virus may be capable of initiating a plaque, but its fecundity is impaired (whereas fecundity of succeeding generations is not) so that the plaque size is ultimately much smaller due solely to the adverse effects of temperature shock in the first generation. These ideas merit further study, including development of theoretical models

that address plaque size formation in VSV, in light of founding-phenotype versus epigenetic factors.

EVOLVED CHANGES IN VIRAL FECUNDITY

We measured evolved changes in viral fecundity using two independent methods. First, we allowed viruses to infect host monolayers in the selective 37°C environment, and examined growth (fecundity) of virus populations in the 6 h following infection. As in the ECS assays, we conservatively used the four evolved virus populations in each group as experimental replicates in these assays. Results (Fig. 2A) for changes in $\log_{10}$ population size showed that delayed-transmission lineages were significantly disadvantaged in mean growth in the 6 h postinfection, relative to viruses evolved under ordinary passage durations (Table S2). These data indicated a trade-off whereby the VSV advantage in ECS was associated with a disadvantage in viral fecundity (see further discussion below).

Second, we measured the average size of plaques produced at 37°C by evolved lineages in the experimental treatments. Here we calculated the mean plaque size of 20 plaques produced by each evolved population, and then used these data in analyses that determined whether plaque size was smaller, on average, for populations evolved under delayed transmission. Results (Fig. 2B) were highly consistent with the above growth-curve data, because average plaque diameter of representative ordinary-transmission viruses was significantly larger (3.03 mm $\pm$ 0.74 SD) than that of delayed-transmission virus representatives (2.13 mm $\pm$ 0.49 SD) (Table S2); the raw data for these assays are shown as a distribution plot in Figure S2.

TRADE-OFF BETWEEN SURVIVAL AND FECUNDITY IN EVOLVED VIRUSES

Taken together, the combined results in Figures 1 and 2 clearly demonstrate that the evolved VSV populations in our study show a trade-off between survival and fecundity, depending on their treatment environment. Based solely on our manipulation of timing-of-transmission (24 vs. 48 h), the evolved viruses, on average, are either advantaged in survival or advantaged in growth, but are not superior in both traits. In particular, the VSV populations passaged under delayed transmission "invested" in an evolved strategy of increased survival at the expense of reduced growth, whereas the ordinary-transmission populations did not diverge in these traits relative to the wild type (Figs. 1 and 2). These results indicated that prolonged time spent outside of cells is an environmental factor sufficient to select for greater virion stability in VSV, echoing earlier results by Elena (2001) who measured growth properties and inferred decay rates of VSV populations evolved under conditions similar to our study. Both studies highlight the need for parasite-evolution theory that implements survival as a potent selective force in predicting how traits such as virulence may evolve (Bonhoeffer et al. 1996; Gandon 1998; Kamo and Boots 2004). Although neither study directly tested theory developed specifically for RNA viruses, the observations are consistent with predictions from life-history theory, which imply fundamental constraints in the ability for evolving populations to simultaneously maximize survival and reproduction (Stearns 1989). Interestingly, the results of Elena (2001) and the current study conflict with the "curse of the pharaoh" hypothesis (Bonhoeffer et al. 1996), which refers to the mysterious death of Lord Carnavon after entering the tomb of the Egyptian pharaoh Tutankhamen (Corelli 1923). Essentially, this hypothesis argues that high survival of free-living parasites should be associated with higher virulence, because parasites that can thrive for long periods outside the host can avoid the cost of an extreme exploitation strategy (Ewald 1987, 1993, 1994; Bonhoeffer et al. 1996; Gandon 1998; Kamo and Boots 2004). However, mathematical models for the curse of the pharaoh often identify ecological conditions that generate the opposite prediction: greater parasite survival that coincides with lowered virulence (reproduction) (Bonhoeffer et al. 1996; Gandon 1998; Kamo and Boots 2004). In particular, Caraco and Wang (2008) merged thinking from life-history and virulence evolution, to suggest that parasites with extreme survival will evolve mechanisms for this trait that should often compromise growth ability. Although the exact mechanism underlying the survival-reproduction trade-off in our study remains unknown, studies such as Dessau et al. (2012) indicate that a single allele substitution in an RNA virus can lead to both improved survival and reduced reproduction, offering a simple antagonistic pleiotropy explanation for the trade-off mechanisms generally suggested by Caraco and Wang (2012) (see further discussion below).

Also, there is continued concern over VSV as an agricultural pathogen particularly in cattle and as a flu-like illness in humans, as well as increasing interest in VSV applications such as a viral vector in vaccination and as a candidate for oncolytic-viral therapy in humans (Lichty et al. 2004). These disease and industrial implications have prompted studies of environmental effects on VSV virion stability (Zimmer et al. 2013), and a logical extension of our experimental evolution studies would be to examine whether further in vitro passage of VSV under delayed-transmission time might lead to compensatory mutations that "break" the trade-off, allowing VSV to maintain added stability while acquiring improved growth at wild-type or greater levels.

OBSERVED MOLECULAR CHANGES AND INFERRED PHENOTYPE–GENOTYPE ASSOCIATIONS

To further distinguish our work from the study by Elena (2001), we conducted whole-genome consensus sequencing of the eight evolved populations (Genbank #KF880907-KF880915), as a

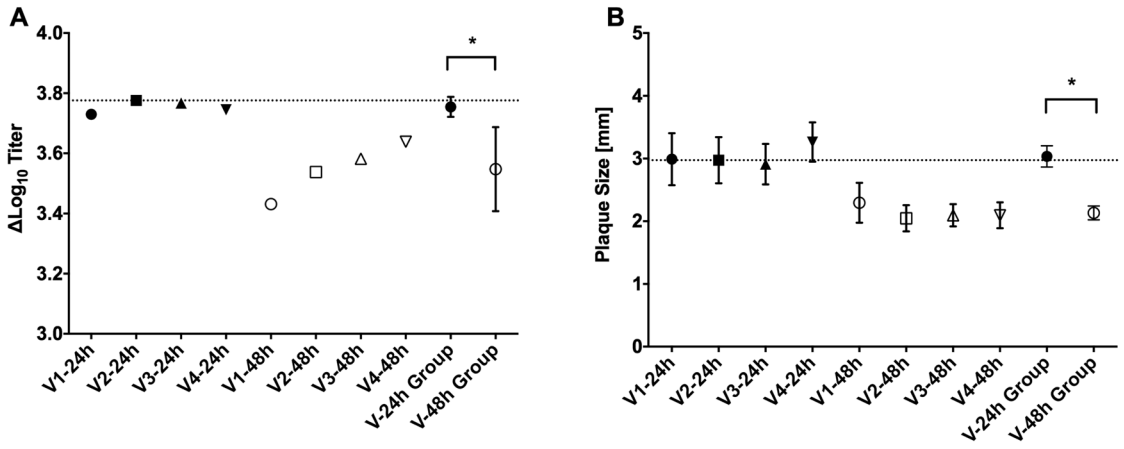

Figure 2. **Mean fecundity of evolved virus populations, measured using two separate assays for viral growth. Ordinary-transmission populations (solid symbols) present measurably higher titers (A) and larger plaque sizes (B), relative to viruses evolved under delayed transmission (open symbols). Statistical analysis of group-wise differences shows that delayed-transmission viruses are significantly disadvantaged in relative fecundity, regardless of the growth assay employed. Mean growth data for the wild-type genotype (dotted lines) are depicted for comparison. See text for details.**

preliminary analysis to infer phenotype–genotype associations according to treatment conditions in our study.

Overall, we identified a total of 30 new (or polymorphic) alleles in the eight evolved populations (20 unique loci); the distributions of these new alleles ranged between zero and seven novel and/or polymorphic loci per population (Fig. 3). The four populations in the ordinary-transmission treatment contained 17 of the 30 observed changes, whereas the four populations in the delayed-transmission treatment showed the remaining 13 changes. Although these numbers are similar, the two treatment groups differed markedly in the polymorphic versus fixed genetic changes observed. In the ordinary-transmission populations, changes were heavily biased toward polymorphisms where the ancestral allele dominated, relative to fixed new alleles (ancestral-dominant polymorphisms were 16 of the 17 observed changes, or 94%). These ancestral-dominant polymorphisms were equally distributed between synonymous and nonsynonymous amino acid changes (eight of each type among the 16 total polymorphisms). Because the ordinary-transmission treatment contained populations with a paucity of fixed substitutions, with one population (V2—24 h) completely lacking any detectable genetic change, this strongly (and unsurprisingly) suggests that the typical 24-h culture conditions did not present a new challenge to the VSV lineages.

In contrast, in the delayed-transmission populations, we observed that fixed substitutions were relatively common (six of 13, or 46%), compared to polymorphic loci (seven of 13, or 54%). Also, the delayed-transmission populations showed nucleotide changes that were relatively biased toward nonsynonymous amino acid substitutions (10 of 13, or 77%), compared to synonymous changes (three of 13, or 23%). Although synonymous substitutions may be important in VSV because these could affect the structure of the single-stranded RNA genome, we note that prior studies that challenge the virus to evolve under novel conditions tend to implicate nonsynonymous changes as most important for adaptation (e.g., Remold et al. 2008). Thus, across the experiment, we observed the expected result that signatures of selection were generally associated with the novel 48-h transfer environment, and not with the typical 24-h passage conditions.

Genomic consensus changes in the ordinary-transmission populations were equally distributed across the five gene-coding regions, particularly accounting for relative coding sequence length (chi-squared test, $P > 0.05$). Similarly, observed changes in the delayed-transmission populations were distributed equally among genes (chi-squared test, $P > 0.05$). However, we noted that in the delayed-transmission populations, neutral distribution predicts that a single mutation (of the 13 total observed) should be located in the VSV matrix (M) protein gene, whereas three mutations were observed. Mutations in the M gene of the delayed-transmission populations were observed every 230 bases on average, which was the highest frequency of changes per base for any gene in the treatment groups. Further, all observed M gene mutations were nonsynonymous. These results suggest that the M gene may have been the more important gene target of selection when viruses experienced delayed transmission, but this idea warrants further investigation.

We attempted to use the genomic data to infer a mechanism whereby increased ECS evolved in the delayed-transmission populations. Based on known function of VSV proteins, a plausible prediction would be increased virion stability via changes in the N (nucleocapsid) and/or G (glycoprotein) genes, with perhaps a role for the M gene because the M protein also contributes to VSV

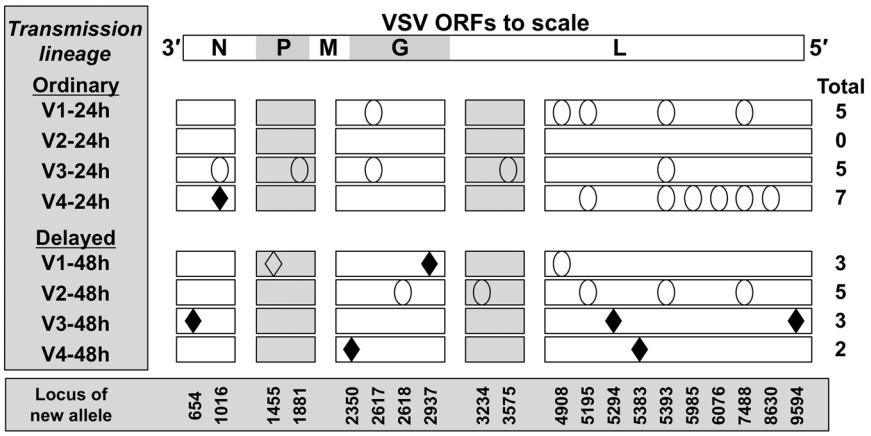

Figure 3. **Consensus genome sequences of the eight experimental lineages, showing observed differences separating them from the common ancestor virus. New alleles (closed diamonds) are noted, as well as polymorphic loci where the ancestral (open circles) or new allele (open diamonds) is dominant.**

structure. However, the molecular data provided no obvious signatures for candidate beneficial mutations that enhance ECS, owing to the lack of convergent substitutions among populations in the delayed-transmission treatment. Although VSV studies containing only four replicate populations per treatment group have sometimes yielded clear evidence of convergence (e.g., Remold et al. 2008), we note that the small number of populations in the delayed-transmission treatment may have limited our ability to observe convergent substitutions. However, as noted above, the delayed-transmission populations were relatively enriched for fixed substitutions that were mostly nonsynonymous, whereas their ordinary-transmission counterparts showed more ancestral-dominant polymorphic loci and roughly equal synonymous and nonsynonymous changes. Taken together, these data further echo the evolution of phenotypic novelty in the delayed-transmission populations, but suggest that there are multiple solutions to gaining increased virion stability, with possible independent roles for the proteins coded by the N, G, and M genes in VSV.

Prior work shows that survival of viruses such as coliphages and VSV can be strongly affected by environmental stressors, and that survival-fecundity trade-offs can be measured (De Paepe and Taddei 2006; Ogbunugafor et al. 2013; Zimmer et al. 2013). However, the underlying mechanisms that drive evolution of such trade-offs have been rarely elucidated. Our previous work in the segmented dsRNA bacteriophage Φ6 (family Cystoviridae) offers a rare example (Dessau et al. 2012). When this phage was subjected to selection to overcome heat-shock degradation, increased thermostability at high temperature was achieved through a convergent point mutation that better stabilized the P5 lytic enzyme that the virus uses for cell entry and exit; however, this molecular change also reduced fecundity, indicating that the allele was antagonistically pleiotropic. In contrast, our results in VSV did not suggest a clear mechanism whereby survival was achieved at the expense of fecundity, even though the evolved trade-off was plainly apparent. Additional work (beyond the current whole-genomics approach) is needed to confirm the mutational spectrum for allele changes in VSV that promote stability at the expense of fecundity, and to conclude that multiple proteins can be altered to produce the same trade-off. With potentially broad mutational spectra underlying the "life-history" trade-off between survival and reproduction in VSV, it suggests that predictions of adaptive outcomes in this RNA virus may be especially difficult (i.e., in contrast to systems such as phage Φ6, where the mutational spectrum is apparently much narrower). We observed a similar lack of convergence and potentially broad mutational spectra underlying adaptation, when VSV was experimentally evolved under varied temperature regimes at ordinary transmission (Alto et al. 2013). Thus, our VSV results also underscore how viruses may possess multiple adaptive solutions to the same environmental problem, which should contribute to the overall success of viruses in the biosphere, especially their impressive biodiversity, adaptive potential, and perhaps even avoidance of extinction compared to other biological entities (Wasik and Turner 2013).

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Mean extracellular survival through time at thermal extremes of the VSV survival niche, for VSV populations evolved under differing transmission.

Figure S2. Distribution plots of raw plaque-diameter measurements, which were used to calculate the summary data shown in Figure 2B.

Table S1. Results from fit least squares model analysis of variance (ANOVA) of extracelluar survival assay at each time point with *transmission* and *time point* as model effects.

Table S2. Results from fit least squares model analysis of variance (ANOVA) of productivity and plaque size experiments.