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# Consequences of host adaptation for performance of vesicular stomatitis virus in novel thermal environments

**Barry W. Alto · Paul E. Turner**

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**Abstract** Although it is widely assumed that the selective advantage of niche specialization drives species biodiversity, some theory suggests that generalists are favored over specialists when environments change unexpectedly. But this idea is rarely tested empirically, and its relevance is unknown for microparasites such as RNA viruses. Due to their small genome sizes pleiotropy is not uncommon in RNA viruses. Therefore, the genetic architectures underlying generalist traits may be indirectly molded by selection to better prepare generalist organisms for growth in new environments. Previously, vesicular stomatitis viruses were evolved to specialize on a single host, or to generalize on multiple hosts. Here we test whether virus generalists arising in the context of host adaptation also perform differently than specialists when viruses grow at novel temperatures. We compared thermal reaction norms of performance, within and among groups of viral specialists and generalists. Results showed that host adaptation was consequential for some fitness traits at novel temperatures due to modification of pleiotropic viral genes. Contrary to theoretical predictions, host generalists were selectively disadvantaged at extreme cool and warm environments. Multi-host adaptation may compromise the evolved thermostability of viral proteins, creating a cost of host generalization when viruses replicate at extreme temperatures.

**Keywords** RNA virus · Ecological generalization and specialization · Trade-offs · Reaction norm

B. W. Alto · P. E. Turner
Department of Ecology and Evolutionary Biology, Osborn Memorial Laboratories, Yale University, 165 Prospect St., New Haven, CT 06520-8106, USA

B. W. Alto (✉)
Illinois Natural History Survey, University of Illinois, 1816 S. Oak St., Champaign, IL 61820, USA
e-mail: alto@illinois.edu

## Introduction

Natural biodiversity is often explained by the presumed tendency for selection to favor specialists with narrow niches, over generalists with broad niches (Rosenzweig 1995; Kassen 2002; Duffy et al. 2006). The typical underlying assumption is that organisms residing in a constant habitat tend to become adaptively specialized, which hinders (or trades-off with) their performance in alternate habitats. Fitness trade-offs can be explained mechanistically by antagonistic pleiotropy (a negative genetic correlation between performance across environments; Levins 1968; Rausher 1984; Lynch and Gabriel 1987; Elena and Lenski 2003), or by the accumulation of neutral mutations that are deleterious in alternative environments (Kawecki 1994). Both mechanisms may genetically constrain the ability for organisms to simultaneously dominate multiple niches (Futuyma and Moreno 1988). Even without assumed trade-offs, theory predicts a cost of generalism because broader niche breadth should slow the rate of evolutionary response; narrower niche breadth should increase the probability of fixing beneficial alleles and decrease the frequency of harmful alleles (reduce mutation load) at mutation-selection equilibrium (Whitlock 1996). Thus, the adage that a "jack-of-all-trades" tends to be a master of none remains popular in evolutionary ecology, due to predicted costs of niche breadth which impede maximal performance in any one niche (Levins 1968; Lynch and Gabriel 1987; see review by Wilson and Yoshimura 1994).

Despite identified costs to generalism, some habitats necessitate that generalists evolve. Adaptive generalization should be especially important in temporally coarse-grained environments where ecological change is complete, deterministic and spans multiple generations. Because such environments lack spatial refuges, individuals in the population must either reproduce or perish. The classic ecological prediction is that a population which experiences few generations in a temporally variable environment should become dominated by generalists: individuals that perform best across all of the habitats encountered (Roughgarden 1972). Assuming genetic trade-offs are common, selection in constant versus temporally variable environments should lead to evolution of very different underlying genetic architectures. For example, generalists selected in environments A and B should differ markedly in genetic architecture relative to specialists selected in environments A or B alone. The reason is that mutations important for trade-offs are unlikely to fix in populations undergoing simultaneous selection in both habitats, because these mutations would impede the process of adaptation via natural selection.

Beyond specific types of coarse-grained environments, are there other conditions where generalists should be favored? Hutchinson (1957) famously referred to the fundamental ecological niche as an *n*-dimensional hypervolume containing all biotic and abiotic variables under which a biological population can thrive. Because specialists by definition occupy a smaller fundamental niche than generalists, Brown (1984) suggested that they are more likely to be adversely affected by reduced habitat availability. This idea implies that specialists should be more often selectively disadvantaged under environmental change (Vazquez and Simberloff 2002), especially when changes are dramatic and extreme (i.e., placing specialists far from their fundamental niche). However, empirical tests of this hypothesis are generally lacking. A few studies suggest that niche generalization improves performance in changing environments; e.g., greater niche breadth seems to protect species of coral reef fish against coral decline (Wilson et al. 2008 and references therein). But to our knowledge no study has compared performance of closely related specialists and generalists at the extremes of the fundamental niche.

Evolution of specialists and generalists is sometimes observed in laboratory tissue-culture experiments where RNA viruses are evolved on a single host-cell type, versus on temporally variable hosts (Weaver et al. 1999; Cooper and Scott 2001; Greene et al. 2005). This differing host adaptation leads to phenotypic divergence: virus specialists improve in fitness on one host at the expense of increased performance on the other host, but alternating-host culture leads to generalists that improve in fitness on both hosts. Similar observations occurred in our experiments with vesicular stomatitis virus (VSV; Turner and Elena 2000). VSV is a small (five genes, ~11-kb genome size) negative-sense ssRNA *Vesiculovirus* in the family Rhabdoviridae that infects livestock mammals and biting insects (Lyles and Rupprecht 2006). We later used whole-genome sequencing to reveal the expected divergence in genetic architectures underlying these adaptive phenotypic observations (Remold et al. 2008). In particular, we showed that the fitness trade-offs suffered by VSV specialists on unselected hosts were due either to fixation of antagonistically pleiotropic alleles or to mutation accumulation, depending on the host used in specialization. By contrast, the genetic architectures of viral generalists differed because these lineages seemed to fix beneficial mutations that afforded simultaneous fitness improvement on both host types (Remold et al. 2008).

Here we examine whether this genetic divergence among VSV populations affects performance when viruses are challenged to grow at new temperatures. We assert that host adaptation is likely to be consequential for performance in unselected habitats, such as novel thermal regimes, because the small genome sizes of RNA viruses tend to require that proteins be multi-functional. Thus, minimal (e.g., point mutation) changes to individual viral proteins governing both cell attachment and cell entry may explain why RNA virus specialization on one host impedes performance—or prevents infection altogether—in an unselected host (e.g., see Duffy et al. 2007). By the same logic, we infer that even subtle genetic-architecture differences among VSV specialists and generalists may cause large growth differences in novel temperatures. Furthermore, many of the observed genetic changes that distinguished host-adapted VSV specialists and generalists occurred in the matrix (M) protein gene (Remold et al. 2008), which is perhaps the most versatile VSV protein, affecting intracellular viral assembly, and envelope budding, as well as controlling the availability of the cellular translation machinery (Flamand 1970; Newcomb and Brown 1981; Her et al. 1997). In addition, the M protein is the only VSV gene where mutations relating to thermal sensitivity have been mapped (Flamand 1970; Gaudier et al. 2002).

We tested whether the divergence between host-adapted VSV specialists and generalists was consequential for performance at novel host temperatures, with the specific prediction that generalists should be favored at thermal extremes. We did so by comparing and contrasting thermal performance curves (Fig. 1) measured for evolved VSV populations, and the wild type ancestor.

## Materials and methods

### Viruses and host cells

VSV populations are a subset of those described by Turner and Elena (2000) (see Remold et al. 2008 for details). Briefly, a single VSV population of Indiana serotype was used to found four lineages in each of three treatment groups, which were passaged every 48 h in tissue-culture habitats. One treatment allowed the test lineages to adapt to growth on human cervical epithelial (HeLa) cells; one treatment allowed adaptation of lineages to

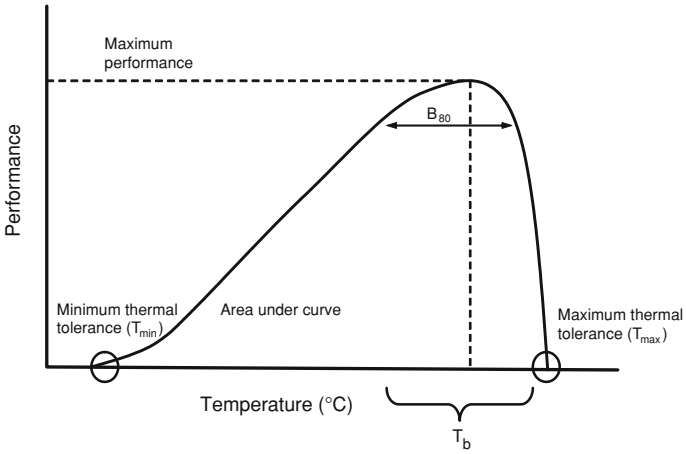


**Fig. 1** Hypothetical performance curve for virus growth (performance) as a function of environmental temperature. The maximum performance occurs at the peak of the curve. $T_b$ describes the performance breadth in temperature where performance among VSV groups is $\geq$80% ($B_{80}$) of maximum. $T_{min}$ and $T_{max}$ describe the minimum and maximum temperature (i.e., tolerance limits) for detectable virus growth. Area under the curve measures total integrated virus performance across the entire thermal reaction norm

canine kidney epithelial (MDCK) cells; and the third treatment involved alternating growth on the cell types. These 12 populations experienced 25 passages each, which is equivalent to $\sim$100 virus generations (four generations per 48 h; Miralles et al. 2000; Turner and Elena 2000; Remold et al. 2008). Throughout the experimental evolution the test lineages experienced an identical incubation temperature of 37°C. The HeLa-adapted and MDCK-adapted lineages improved in fitness (relative to the common ancestor) at the expense of performance increase in the unselected host environment; therefore, these lineages were defined as host specialists (Turner and Elena 2000; Remold et al. 2008). In contrast, the lineages evolved on alternating hosts improved in fitness in both habitats and were defined as host generalists (Turner and Elena 2000; Remold et al. 2008).

The current study used the 24th-passage ($\sim$96-generation) derived populations. Each specialist population was taken from $-$80°C storage, and subsequently passaged once on the selected host. Similarly, generalist populations underwent a single HeLa passage. These passaged isolates were then titered and used in experiments. We also studied a plaque-purified clone from the population that served as an ancestor in the study by Turner and Elena (2000). This clone (hereafter referred to as wild type) enabled us to compare thermal performance and niche breadth of derived VSV populations and an unevolved clone. The inclusion of the ancestor was for sake of completeness, as it was unknown how the common ancestor performed across the temperature gradient.

## Culture conditions and temperature performance assays

Unless otherwise noted, virus infections were conducted using monolayers of baby hamster kidney (BHK) cells, and the culture methods of Turner and Elena (2000). Tissue culture flasks (25 $cm^2$) were seeded with $\sim 7.5 \times 10^5$ cells/ml, and Dulbecco's modified Eagle's minimum essential medium (DMEM) or Leibovitz L-15 medium containing 10% fetal bovine serum (FBS), and antibiotics penicillin and streptomycin. After 24 h incubation at 37°C in a 5% $CO_2$ atmosphere, cells achieved confluence and a final size of $\sim 1.12 \times 10^7$

total cells, on average. A 200-µl inoculum containing $2.8 \times 10^4$ viruses in DMEM was then added to the monolayer. Experimental flasks were gently rocked every 15 min during 1 h incubation to allow viral attachment and entry. Then, 5 ml of medium was added to each flask and flasks were moved to an incubator preset to the assay temperature. Thus, all assayed populations featured identical numbers of founding individuals, and were allowed to establish 1-h infection under the identical thermal condition of 37°C. After 48 h of viral growth at the assay temperature, supernatant containing virus was removed from the flask and aliquots stored at −80°C. Virus titers were gauged by replicate ($n = 2$) plaque assays using monolayers of BHK, the original host for all derived VSV populations and the wild type (Turner and Elena 2000). Each plaque is assumed to have originated from a single infecting virus. Although the subsequent host adaptation sometimes led to decreased fitness (relative growth rate) on BHK (Turner and Elena 2000), this permissive cell type does not bias against titer enumeration for any of the derived populations (*unpublished data*).

Temperatures were chosen based on results from preliminary experiments, which identified the full range of temperatures where at least some of our VSV strains could be recovered at 48 h (*unpublished data*). Chosen assay temperatures were 14, 18, 23, 28, 33, 37, 42, and 44°C, analyzed in a single temporal block (4 virus groups × 4 replicates/group × 8 temperatures = 128 flasks total). Actual incubator temperatures (mean ± SE) were monitored (HOBO® data logger, Onset, Bourne, MA), and shown to be 13.49 ± 0.092, 18.01 ± 0.003, 23.65 ± 0.011, 29.65 ± 0.014, 33.76 ± 0.006, 37.31 ± 0.049, 42.67 ± 0.022, and 43.66 ± 0.017°C.

## Cell counts to gauge virus productivity

Healthy BHK cells remain attached to the flask surface during growth and division, but VSV infection causes cytopathic effects whereby cells become detached from the surface and float in the medium. Following the temperature assays, media was aspirated from infection flasks followed by a 3 ml rinse with phosphate buffer solution (PBS) (1x) to remove infected and dead cells, and flasks were stored at −20°C. This manipulation left undisturbed the cells that were presumably healthy or in the early stages of VSV infection. These remaining cells reflect unused resources during virus population growth, useful for gauging virus resource utilization. To do so, stored flasks were thawed, and 5 ml DMEM added. Cells were gently detached using a cell scraper, followed by vigorous pipetting. Cell densities (cells/ml) were then estimated by microscopic examination using a hemocytometer. We recognize that vigorous pipetting may result in fragmentation of some cells which may result in liberal estimates of cell counts. Regardless, we are only concerned with the relative number of cells remaining after infection between the virus groups.

## Statistical analyses

Thermal performance curves, plots of performance (virus growth) as a function of temperature (Huey and Stevenson 1979; Izem and Kingsolver 2005), for individual replicates of VSV groups were obtained by fitting empirical data with a standard 4th degree polynomial using Table Curve (version 5.01; Systat Software, Inc. 2002). These fits accounted for a thermal reaction norm with an asymmetrical distribution attributable to negative skewness, because biological functions tend to decrease more rapidly at higher temperatures, than at lower ones. Subsequently, we measured each test strain's maximum performance, area under the curve, and performance breadth (Fig. 1). Area under the curve represents total integrated performance (e.g., Palaima and Spitze 2004; Palaima 2007). The

performance breadth was measured as the range, in temperature °C, where the test strain showed ≥80% maximum performance (e.g., Angilletta et al. 2002). Thermal tolerance limits were measured as the extreme high ($T_{max}$) and low ($T_{min}$) temperatures where virus production was measurable. A one-way multivariate analysis of variance (MANOVA) was calculated to determine VSV group effects on performance measurements (maximum performance, area, breadth) (Scheiner 2001; SAS Institute 2002). Subsequently we used univariate Tukey–Kramer multiple comparisons among group least-squares means for pairwise comparisons of performance measurements between VSV groups.

Two-sample *t*-tests (one-tailed) were used to compare VSV groups at thermal extremes (i.e., 14 and 42°C) and to address the specific hypothesis that generalist viruses are advantaged relative to specialists at environmental extremes. We chose 42°C as the upper thermal extreme because all but one test strain could be titered at this temperature, allowing comparisons not possible at 44°C where several extinctions occurred (see Results). Sequential Bonferroni adjustments for experimentwise $\alpha = 0.05$ were made for multiple tests.

We analyzed the number of host cells remaining after VSV infection using a two-way ANOVA with VSV group, temperature, and interaction as effects. We used a rank transformation (SAS Institute 2002, PROC RANK, PROC ANOVA) because these data did not meet the assumption of normality (Conover and Iman 1981). Significant effects were further analyzed by pairwise contrasts with a correction for experimentwise $\alpha = 0.05$ (SAS Institute 2002). Results did not differ between the rank transformed and untransformed data. Thus, these data appear to be robust to departure in normality. For ease of interpretation, we only present untransformed data.

Product-moment correlation coefficients ($r_{1,2}$) were used to describe the relationship between virus growth and the number of remaining host cells after VSV infection (pfu/ml versus cells/ml). Sequential Bonferroni adjustments for experimentwise $\alpha = 0.05$ were made for multiple tests. These analyses enabled us to test the strength of the relationship, presumably negative, between VSV production and resource utilization, as measured by the number of remaining cells at the end of the experiment.

## Results

We used VSV lineages evolved on either of two host environments (HeLa or MDCK cells) defined as specialists, and those evolved on alternating hosts defined as generalists (Turner and Elena 2000; Remold et al. 2008). Here we tested whether the prior evolution and genetic divergence also caused the groups of virus populations to perform differently in novel thermal environments.

### Wild type VSV traits

Replicated ($n = 4$) 48-h assays were used to measure performance of the wild type virus clone across eight temperatures (14, 18, 23, 28, 33, 37, 42, and 44°C), to estimate the ancestral performance curve (reaction norm; Fig. 1) and thus identify the ancestral thermal niche. These data produced the genotype's LS means for maximum performance, area under the curve, and breadth ($T_{80}$) listed in Table 1. Virus growth was detected in seven of the eight assay temperatures examined; the most extreme high temperature (44°C) resulted in extinction of all assay replicates (Fig. 2). At 42°C three of the four replicates grew with a mean of 4.13 $\log_{10}$ virus/ml, whereas the 14°C temperature resulted in a mean growth of

**Table 1** Performance measurements of wild type and evolved VSV populations along a thermal reaction norm

| Virus | Area | Max. performance | Breadth | $T_{min}$ | $T_{max}$ |
|---|---|---|---|---|---|
| Wild type | 236.36 | 10.47 | 15.68 | 4.14 | 0 |
| Wild type | 241.47 | 10.55 | 16.45 | 3.54 | 1.57 |
| Wild type | 246.22 | 10.37 | 17.71 | 3.74 | 5.27 |
| Wild type | 246.96 | 10.43 | 17.51 | 3.58 | 5.55 |
| LS mean | 242.75 | 10.46 | 16.84 | 3.62 | 4.13 |
| SE | 2.21 | 0.08 | 0.55 | 0.16 | 0.85 |
| MDCK | 255.31 | 10.67 | 16.58 | 5.30 | 6.04 |
| MDCK | 248.79 | 10.58 | 15.21 | 4.64 | 6.60 |
| MDCK | 249.16 | 10.23 | 18.55 | 4.52 | 5.15 |
| MDCK | 253.30 | 10.45 | 17.04 | 5.30 | 6.99 |
| LS mean | 251.64 | 10.48 | 16.84 | 4.94 | 6.20 |
| SE | 2.21 | 0.08 | 0.55 | 0.14 | 0.74 |
| HeLa | 227.13 | 10.11 | 14.69 | 4.56 | 2.35 |
| HeLa | 233.03 | 10.39 | 14.48 | 4.76 | 2.10 |
| HeLa | 229.96 | 9.92 | 15.50 | 4.62 | 3.20 |
| HeLa | 241.44 | 10.09 | 17.09 | 5.15 | 5.68 |
| LS mean | 232.89 | 10.12 | 15.44 | 4.77 | 3.33 |
| SE | 2.21 | 0.08 | 0.55 | 0.14 | 0.74 |
| Generalist | 241.49 | 10.54 | 15.32 | 4.20 | 4.18 |
| Generalist | 236.88 | 10.33 | 16.10 | 4.11 | 1.68 |
| Generalist | 242.26 | 10.37 | 16.54 | 4.32 | 3.80 |
| Generalist | 239.13 | 10.16 | 17.31 | 4.48 | 2.48 |
| LS mean | 239.94 | 10.35 | 16.32 | 4.28 | 3.04 |
| SE | 2.21 | 0.08 | 0.55 | 0.14 | 0.74 |

Individual performance measurements and least squares (LS) means and standard errors (SE) are shown for each virus strain (wild type, generalist, Madin-Darby canine kidney (MDCK)-specialist, human epithelial carcinoma (HeLa)-specialist) (PROC GLM, SAS Institute 2002). Performance measures include; Area under the curve, maximum performance ($\log_{10}$ virus/ml), breadth ($B_{80}$, range of temperature), minimum thermal tolerance ($T_{min}$, $\log_{10}$ virus/ml), and maximum thermal tolerance ($T_{max}$, $\log_{10}$ virus/ml). Here we describe performance at $T_{min}$ and $T_{max}$

3.62 $\log_{10}$ virus/ml (Table 1). We concluded that the eight assay temperatures encompassed the thermal niche of the wild type.

## Performance comparisons among specialists and generalists

The prior host adaptation treatments led to differing genetic architectures among the three groups of evolved viruses (HeLa-specialists, MDCK-specialists, HeLa-MDCK-generalists) (Remold et al. 2008). Despite these treatment-induced differences, all populations experienced common 37°C incubation during their prior evolution. To examine whether or not the prior selection generally affected performance in novel temperatures, we compared reaction-norm results for the evolved VSV groups. We compared and contrasted thermal performance data for the three evolved VSV groups. We found an overall significant

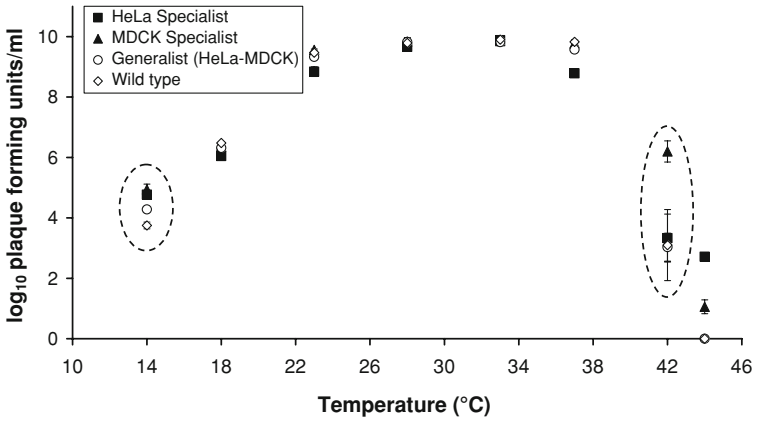


**Fig. 2** Least-squares mean (SE) virus production (performance) for vesicular stomatitis virus groups (HeLa-specialists, MDCK-specialists, HeLa-MDCK generalist, Wild type) along a thermal reaction norm. Viruses were adapted to single hosts (specialists) or alternating hosts (generalists). Means enclosed by dashed ellipses show significant or marginally significant differences in mean virus production between generalist and specialist (as a group) viruses. Plaque forming units/ml refers to the number of viruses/ml

multivariate effect of performance measurement differences between these VSV groups (MANOVA, Pillia's trace $6,16 = 1.38$, $P = 0.002$). Univariate follow up comparisons showed MDCK-specialists had significantly greater maximum performance than HeLa-specialists ($P = 0.03$, Table 1). Maximum performance was similar for all other comparisons between evolved viruses ($P > 0.05$, Table 1). MDCK-specialists had significantly greater area under the curve relative to HeLa-specialists ($P = 0.0003$, Table 1) and to generalists ($P = 0.01$, Table 1). Area under the curve was similar for the comparison between HeLa-specialists and the generalists ($P > 0.05$, Table 1). All evolved viruses had similar performance breadths (all $P \geq 0.32$, Table 1). We concluded that HeLa specialization reduced maximum performance relative to MDCK specialists. Also, MDCK specialists showed greater overall performance (area under curve) relative to other evolved viruses (HeLa-specialists, generalists) over the range of temperatures.

## Thermal tolerance comparisons among specialists and generalists

To test whether host adaptation altered thermal tolerance we analyzed growth of evolved specialists and generalists at thermal extremes. All evolved viruses were detectable after 48 h at 14 and 42°C, indicating common $T_{min}$ and $T_{max}$ (Fig. 2). All MDCK specialists and one HeLa-specialist population had measurable growth at 44°C (Fig. 2). All other virus populations went extinct at 44°C. A Kruskal–Wallis test showed that the number of specialists which positively grew was significantly greater than generalists with no growth ($H = 6.1621$, $P < 0.025$), and relative to other treatments overall (i.e., generalists plus wild type, $H = 3.9286$, $P < 0.05$).

Performance differences between specialists (HeLa- and MDCK-specialists combined) and generalists were significant at extreme cool temperature ($t_s = 3.18$, $df = 10$, $P = 0.0049$), and marginally significant at extreme warm temperature ($t_s = 1.62$, $df = 10$, $P = 0.067$) (Fig. 2). Similar significant effects were found for MDCK- and HeLa-specialists alone (14°C: MDCK-specialist $t_s = 2.95$, $df = 6$, $P = 0.0128$; HeLa-specialist $t_s = 3.17$, $df = 6$, $P = 0.0097$), except for HeLa-specialists at 42°C (MDCK-specialist

$t_s = 4.50$, $df = 6$, $P = 0.0020$; HeLa-specialist $t_s = 0.30$, $df = 6$, $P = 0.3872$). There appeared to be greater variance in performance among all VSV groups at the 42°C extreme (Fig. 2); a test for differences between variances showed significantly greater variance among all VSV groups at 42°C relative to the 37°C selection temperature ($F = 15.28$, $P < 0.001$). However, differences in variances were not significant at the 14°C extreme relative to 37°C ($F = 1.33$, $P > 0.05$). We concluded that specialization on either HeLa or MDCK cells predisposed viruses to perform better than generalists at extreme low and high temperatures. Thus, our data suggested that host generalization was costly for performance in novel thermal extremes.

We considered the possibility that superior performance and temperature tolerance of MDCK specialists was biased by using BHK cells to conduct the reaction-norm experiments. That is, MDCK specialists may have been predisposed to perform well on BHK cells regardless of assay temperature because they improved relative to the ancestor on these cells, whereas the other experimental lineages did not (Turner and Elena 2000). To examine this idea, we performed a follow-up experiment in which all 12 evolved populations were assayed for 48-h growth only at the extreme 14 and 42°C temperatures where MDCK specialists showed a clear advantage. These assays were performed by allowing viruses to infect HeLa or MDCK cells, rather than the BHK-cell infections used in our other experiments. Prior to their use in the experiment, virus groups were grown on their respective host cell types used in the selection experiment (HeLa or MDCK cells; Turner and Elena 2000). Generalists were grown on both HeLa and MDCK cells in order to test for the possibility of host-specific growth responses. Virus production did not differ among generalists grown on either HeLa or MDCK cells so they were treated as a single group in the subsequent analysis (Two sample *t*-tests, two-tailed; all $P \geq 0.47$). An ANOVA showed significant effects of temperature and VSV group (Table 2). All other effects were not significant (Table 2). The temperature effect showed significantly greater virus production at 14 than 42°C (LS mean ± SE, 14°C, 4.87 ± 0.09; 42°C, 2.31 ± 0.09). The VSV group effect showed that MDCK specialists had significantly greater virus production relative to both the HeLa-specialist and generalists (LS mean ± SE, MDCK-specialist, 3.84 ± 0.13, HeLa-specialist, 3.43 ± 0.13, generalist, 3.49 ± 0.09). The lack of a significant VSV group × host cell type interaction (Table 2) supports the idea that the superior performance of MDCK specialists was not biased by host type.

**Table 2** Analysis of variance for effects of virus production (performance) for vesicular stomatitis virus groups (HeLa-specialists, MDCK-specialists, HeLa-MDCK-generalists) grown on HeLa and MDCK host cells at 14 and 42°C

| Source | *df* | *F* | *P* |
|---|---|---|---|
| Temperature (temp) | 1 | 345.74 | <0.0001 |
| VSV group (VSV) | 2 | 2.98 | 0.05 |
| Host cell (host) | 1 | 1.01 | 0.31 |
| Temp × VSV | 2 | 0.29 | 0.75 |
| Temp × host | 1 | 2.68 | 0.10 |
| VSV × host | 2 | 0.33 | 0.72 |
| Error *df* | 54 | | |

Initially we tested for the three-way interaction between virus group, host cell type, and temperature. The interaction was not significant so we omitted it and re-ran the analyses

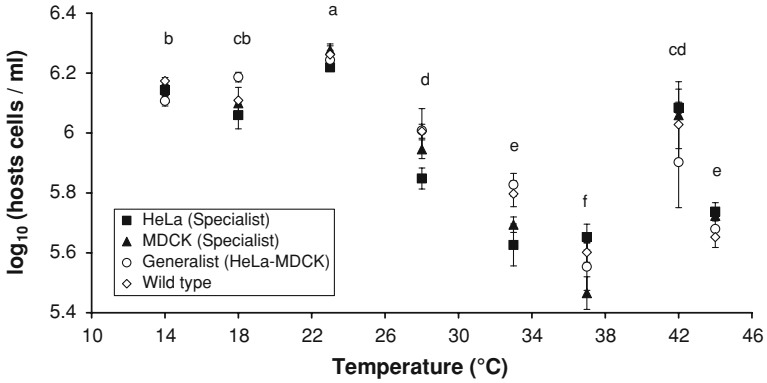


**Fig. 3** Least-squares mean (SE) remaining host cells (resources) after infection with vesicular stomatitis virus groups (HeLa-specialists, MDCK-specialists, HeLa-MDCK generalist, Wild type). Viruses were adapted to single hosts (specialists) or alternating hosts (generalists). Pooled across all vesicular stomatitis virus treatments, means followed by different letters were significantly different from each other

## Host cell usage by ancestral and evolved viruses

To test whether ancestral and evolved viruses differ in their host cell usage we compared the number of remaining resources (host cells) to virus production at the end of the experiment. Product-moment correlations showed significant relationships between virus growth and the number of remaining host cells for VSV groups overall ($P < 0.01$, $-0.4202$), and for all replicates within a group (all $P < 0.01$; HeLa-specialist $= -0.5616$; MDCK-specialist $= -0.5848$, wild type $= -0.4931$), except for generalists ($-0.1467$, $P > 0.05$). Correlations excluded the 44°C treatment because most replicate populations went extinct. Because all instances showed an inverse relationship between virus production and the number of remaining host cells, these results confirmed that cell counts served as useful indicators of virus production in the various thermal conditions. For the thermal reaction norm experiment, remaining-cell counts were significantly affected by assay temperature ($F_{7,93} = 76.29$, $P < 0.0001$). VSV group ($F_{3,93} = 0.63$, $P = 0.60$) and the interaction of group × assay temperature ($F_{21,93} = 1.50$, $P = 0.10$) did not have significant effects on remaining cells. Temperature effects, pooled across VSV groups, showed significantly greater cell counts at 23°C than at all other temperatures (Fig. 3). In general, lower temperatures (e.g., 14–23°C) resulted in the greatest number of remaining cells and there was a consistent and significant decline in cell counts at lower to moderate temperatures (23–37°C) (Fig. 3). Additional increase in temperature (e.g., 42°C) produced cell counts similar to, or exceeding, those at moderate temperatures (28–37°C) (Fig. 3). Further temperature increases (e.g., 44°C) resulted in significantly lower cell counts than most other conditions (Fig. 3). Temperatures much higher than 37°C are likely to start causing cell death. We concluded that temperature altered resources (host cells) used by viruses, but that the viruses did not measurably differ in resource use.

## Potential importance of population genetic variation on conclusions

Due to their characteristically high mutation rates, VSV populations are assumed to contain substantial genetic variation. The duration of the thermal assay (48 h) may have allowed several generations of viral replication in some experiments (Miralles et al. 2000). Thus, we

hypothesized that growth differences among VSV groups at thermal extremes (14, 42°C) may be due to enrichment of mutants that happened to grow well at these temperatures, within the polymorphic populations. To test this hypothesis we took stored virus samples from assays at 14, 37, and 42°C, and challenged them to grow for 48 h at a common 14°C temperature. Support for the hypothesis would be the observation that prior 14°C growth enhances subsequent 14°C growth, relative to 37 and 42°C growth. All replicates within a VSV group were assayed at identical low MOI, but titer differences among stored samples precluded use of an identical MOI of 0.01 across groups (HeLa specialists, MOI = 0.00011; MDCK specialists, MOI = 0.01; generalists, MOI = 0.00043). Relevant comparisons to test this hypothesis involve comparing virus production from samples previously maintained at the different temperature treatments (14, 42, and 37°C). We therefore did not conduct comparisons between VSV groups. In addition, the wild type clone (MOI = 0.00024) was included as a control because we expected its greatly reduced genetic variation would not lead to significantly different treatment outcomes. The generalist group and wild type contained only threefold replication in these assays.

Separate one-way ANOVAs were used for each VSV group to test whether prior growth temperature affected subsequent growth at 14°C. Previous growth at 14, 37, and 42°C did not significantly affect subsequent growth at 14°C for MDCK specialists ($F_{2,7} = 2.52$, $P = 0.1501$), generalists ($F_{2,7} = 3.87$, $P = 0.0739$), or wild type ($F_{2,7} = 0.12$, $P = 0.8860$) (Fig. 4). For HeLa specialists, previous growth at 14°C enhanced subsequent growth at 14°C, relative to 42°C, but not 37°C. Also, previous growth of the HeLa-specialists at 37°C resulted in significantly greater growth at 14°C, than did previous growth at 14 and 42°C (Fig. 4). Although not significant, each evolved VSV group (but not the wild type) showed a trend for enhanced growth at 37°C, relative to 14 and 42°C (Fig. 4). We concluded that previous growth at 14, 37, and 42°C did not alter subsequent performance at 14°C for MDCK specialists and the generalists. Thus, we do not find support for the hypothesis that differences in viral growth between MDCK specialists and the generalists at thermal extremes (14, 42°C) are due to mutant enrichment. In contrast,

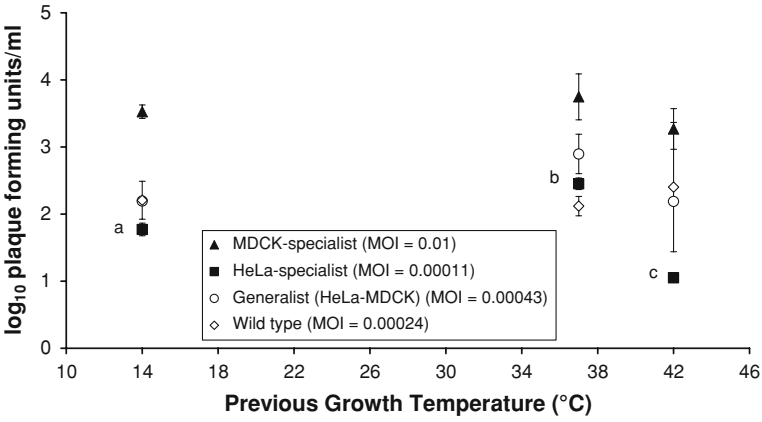


**Fig. 4** Least-squares mean (SE) virus production (performance) at 14°C for vesicular stomatitis virus groups (HeLa-specialists, MDCK-specialists, HeLa-MDCK generalist, Wild type) previously grown along a thermal reaction norm (x-axis). Viruses were adapted to single hosts (specialists) or alternating hosts (generalists). Only HeLa-specialist means followed by different letters were significantly different from each other. Multiplicity of infection (MOI; ratio of viruses to cells) used to initiate the experiment was uniformly low, but differed for each VSV group. Plaque forming units/ml refers to the number of viruses/ml

HeLa specialists previously grown at 14°C showed better performance at this temperature, relative to those grown at 42°C, suggesting some importance of mutant enrichment for these viruses in our study.

## Discussion

We examined whether prior experimental host-adaptation that led to genetic divergence in host-use traits, affected subsequent performance of VSV populations across an identified thermal niche. Our results showed that the host type(s) on which viruses specialized or generalized held consequences for thermal performance traits estimated via reaction norms.

### Superior performance of MDCK-specialists in novel temperatures

One major result of our study was that MDCK-adapted specialists performed as well or better than other evolved viruses and the wild type, in terms of measured thermal traits. Because both HeLa-adapted specialists and generalist viruses tended to perform worse than MDCK specialists, these data strongly suggested that the molecular substitutions associated with fitness improvement on HeLa cells at 37°C, somehow constrained improved performance across the thermal gradient. Interestingly, this outcome did not depend on the total time that viruses spent in the HeLa selective environment: 100% for HeLa-specialists versus 50% for the host-alternating generalists. These results echo some of our previous observations: both adaptation to HeLa cells and to fluctuating hosts led to reduced competitive ability on the original BHK host cells, whereas MDCK specialization caused correlated fitness improvement on BHK (Turner and Elena 2000). Thus, both the previous and current studies showed that virus adaptation to one portion of the niche (HeLa cells) was detrimental for future success in unselected habitats. Unlike many other cells used in tissue culture studies, HeLa are cancer cells and they are not derived from kidney tissues like BHK and MDCK cells. Thus, HeLa hosts may pose a unique set of environmental challenges.

Mutations shared between the HeLa-specialist and generalist viruses cannot be responsible for these results. The logic is that 67 new alleles occurring at 34 loci were observed among the 12 total evolved populations, and only a single mutation ($C_{4180} \rightarrow A$) in the G (glycoprotein) gene was shared between a pair of populations in these two groups (see Fig. 2 of Remold et al. 2008); the G protein is important in attachment to cellular receptors and in cell entry. Rather, one likely possibility is that various changes in the M (matrix) protein and/or L (large) protein genes happen to be responsible, because the HeLa-specialist and generalist viruses underwent 19 total allelic changes in these genes that differed from the four observed changes occurring in the MDCK-specialists (Remold et al. 2008). The M protein provides many varied functions, affecting viral assembly within the cell and envelope budding, and controlling the availability of the cellular translation machinery (Flamand 1970; Newcomb and Brown 1981; Her et al. 1997). The L protein is the viral RNA-dependent-RNA polymerase, which participates with P (phosphoprotein) to form the transcriptase-replicase complex. Thus, alteration of these versatile proteins during the prior adaptation to HeLa cells may have inadvertently affected their function when viruses grow in novel temperatures. Further experiments are needed to confirm whether mutations important for VSV adaptation to HeLa cells are also antagonistically pleiotropic for fitness on BHK cells, and/or growth in certain temperatures.

As an alternative explanation, we considered that the superior performance of MDCK specialists in novel temperatures resulted from a bias in using BHK cells to conduct the reaction-norm experiments. However, the superior performance of MDCK specialists at extreme temperatures was not dependent on infection occurring in the original BHK host or in the selected host, MDCK. Rather, the advantage extended to MDCK cells and even to infection of HeLa cells, a host-type in which MDCK specialists did not improve in fitness relative to the ancestor (Turner and Elena 2000; Remold et al. 2008). These data strongly suggested that genotype × host-cell-environment interactions played a minimal (if any) role in the observed superiority of MDCK specialists in our study, and that experiments conducted on BHK cells did not bias in favor of these viruses.

As a final indicator of the apparent superiority of MDCK-specialist viruses in our experiments, consider the resource-utilization assays we performed. Overall, these assays demonstrated that resource utilization did not differ among VSV treatments across temperatures (i.e., we observed no significant VSV treatment effect or VSV treatment × temperature interaction). Thus, these results suggest that the MDCK-specialists may be more efficient in host utilization compared to the other derived VSV lineages and the wild type, because their higher performance measures were attained despite using similar numbers of resources (cells) as their counterparts.

## Correlated responses to selection within virus groups

Specialized and generalized adaptation involving HeLa cells likely explains the relatively weaker performance of these derived VSV strains in novel temperatures. However, the generally superior thermal performance of MDCK specialists regardless of cell type suggests that mutations for MDCK specialization may be responsible. More work is needed to address this hypothesis, but we note that the available molecular data lend little support. The four MDCK-adapted lineages showed little evidence of adaptive convergence during their prior evolution: 24 total mutations were observed among the lineages in this group, and none of these changes occurred in all four populations (Remold et al. 2008). Furthermore, one of the MDCK specialist lineages had only two fixed mutations separating it from the ancestor, and both of these mutations also fixed in three out of the four generalist lineages. Therefore, specific molecular substitutions for MDCK specialization almost certainly do not account for the current findings on superior performance of MDCK-adapted populations across the thermal niche.

Regardless of the underlying mechanism(s), our study indicates that RNA viruses selected in one context experience mutations consequential for performance in unselected areas of the fundamental niche. To our knowledge, no previous virus study has looked for effects of biotic selection on viral performance in novel abiotic contexts. Rather, virology studies more often concern effects of host selection on performance in new biotic contexts. For example, most of the prior experiments on viral adaptation to new hosts have tested whether host-selection regimes led to positively or negatively correlated performance in the original and unselected hosts, and all possible outcomes have been observed (Greene et al. 2005; Zárate and Novella 2004; Cooper and Scott 2001; Turner and Elena 2000; Novella et al. 1999; Weaver et al. 1999). Whereas viral adaptation studies often focus on biotic components of the fundamental niche, temperature-selection studies necessarily involve abiotic components. Temperature is an easily manipulated continuous variable, allowing researchers to explore whether selection (or maximal performance) at one temperature affects performance at other temperatures, or alters niche breadth (Huey and Hertz 1984; Scheiner et al. 1989; Bennett and Lenski 1999; Gaston and Spicer 2001; Holder and

Bull 2001; Preest and Pough 2003; Palaima and Spitze 2004; Angilletta et al. 2006). For example, Knies et al. (2006) showed that prior selection of bacteriophage G4 at elevated temperatures altered the thermal reaction norm, but in ways specific to the particulars of the thermal selection regime (see also Holder and Bull 2001).

Unlike many previous studies, we have full knowledge of the fixed substitutions (and, hence, genetic divergence) separating the VSV populations in our study, because these lineages were subjected to whole-genome sequencing (Remold et al. 2008). These molecular data showed that the four replicate populations within each of the three treatment groups (HeLa specialists, MDCK specialists, HeLa/MDCK generalists) sometimes fixed identical beneficial mutations, due to convergence among lineages evolving independently under an identical environmental challenge (Remold et al. 2008). However, lineages within each treatment group also diverged somewhat, due to differing molecular substitutions separating them from the common ancestor (Remold et al. 2008; see Travisano et al. 1995 for a similar example using *Escherichia coli*, inferred through phenotypic data). Despite these within-group differences, we found that among-group divergence was more important for determining phenotypic performance of evolved lineages in novel thermal environments. Recall that the four virus lineages within a treatment group served as the independent replicates in our reaction-norm experiments. This design allowed us to conservatively test for effects of prior host adaptation on thermal performance, because the replicate lineages were known to have undergone some genetic divergence (Remold et al. 2008). Despite these genetic differences, we still observed statistically significant effects of treatment group on thermal performance. Similar results have been observed in non-virus systems. For example, *Drosophila melanogaster* populations selected for increased desiccation resistance showed correlated responses to resistance of other environmental stressors such as heat shock, and starvation (Hoffman and Parsons 1989a, b). This result was attributed to evolution of reduced metabolic energy expenditure in desiccation-selected flies, which provided beneficial resistance to a variety of unselected stressors. In contrast, we attribute our results to the general importance of pleiotropy in RNA viruses, especially selective modification of multi-functional proteins in one environmental context which proved circumstantial for growth in novel abiotic settings.

## Evidence for costs of host generalization in novel thermal environments

MDCK-specialist viruses showed superior performance in many of our experiments, but we also observed findings that indicated generalists were disadvantaged relative to both groups of specialist viruses. In particular, generalists seemed to perform worse under the most extreme regions of the thermal niche. The upper portion of the temperature niche was analogous to a "knife's edge" where small changes in temperature resulted in more dramatic phenotypic responses, relative to our observations at moderate temperatures. This statement is supported by (1) incremental changes at elevated temperatures drove $\sim$67% of the test strains extinct (i.e., 10 of 15 strains which grew at 42°C went extinct at 44°C), and (2) variance in phenotypic responses were significantly greater at a higher stressful temperature than at lower temperatures (i.e., greater variance at 42 than 37°C; see also Cooper et al. 2001 for a similar observation in *E. coli*). Extreme thermal-maximum tolerance limits were significantly greater for specialists relative to other VSV strains including generalist viruses alone, although these results were stronger for MDCK-specialists than HeLa-specialists. That is, only some populations of specialists were able to

survive at 44°C, whereas all generalists and many other virus strains went extinct under this condition. Thus, prior host specialization, but not generalization, caused an extension of the upper limit of the thermal niche. Although the physiological basis is unknown, greater protein stability of viral specialists at high temperatures is consistent with the observed results (e.g., Bull et al. 2000). Evidence for altered thermal tolerance limits has also been observed in temperature adaptation of *E. coli* ("stepping stone" and "sliding niche" hypotheses, Mongold et al. 1996, 1999) and of *Drosophila* (Huey et al. 1991; Watson and Hoffmann 1996).

Evolutionary ecology models (e.g., Lynch and Gabriel 1987) suggest trade-offs may exist for tolerance adaptation. Although the scientific literature does not extensively address how generalists and specialists should differ in novel habitats, our data counter the idea that generalists are advantaged at niche limits (Levins 1968; Kassen 2002). Instead, we found the opposite: generalists performed poorer at thermal extremes than specialists. These results are potentially far-reaching. For example, viral emergence on new hosts may be promoted by extreme-temperature tolerance. This idea has been proposed for cold-adapted genotypes of Influenza-A Virus which may benefit from increased survival outside of the preferred niche, allowing these variants to more often contribute to disease progression and perhaps flu epidemics (Hatta et al. 2007). Specifically, highly pathogenic H5N1 influenza variants grow better and are more tolerant of low-temperature survival in the upper respiratory tract of mammals due to an amino acid change in the PB2 protein, which may facilitate adaptation of avian H5N1 to human-to-human transmission (Hatta et al. 2007). In the current study, our results suggest that temperature tolerance may be altered via indirect selection on other virus traits involved in host specialization versus generalization.

Experiments on host-use adaptation in RNA viruses do not always conform to the widespread assumption that generalists adapt more slowly than specialists. Rather, viruses which adapt to multiple hosts (a broad niche) sometimes achieve the same performance increase as viruses adapting to only a single one of the hosts (Novella et al. 1999; Weaver et al. 1999; Turner and Elena 2000). These observations indicate that spending half the evolutionary time in a selective niche does not always create a cost of adaptation. Presumably these observations occur because RNA viruses are not mutation-limited; the characteristically error-prone replication of these microbes allows them to "find" the mutations that are simultaneously beneficial across multiple niches, whereas DNA systems may be more prone to the expected constraint (Turner and Elena 2000). Judging from these laboratory results, one might then ask: Why do naturally occurring RNA viruses tend to be highly specific to their hosts, given that generalist variants are seemingly not strongly selected against? One possibility is that wild type viruses do not typically experience selection which causes them to fix mutations of benefit across variable hosts (i.e., multi-host selection occurs so rarely that viruses tend not to generalize). Alternatively, RNA viruses may tend to specialize because evolution of increased niche breadth may incur heretofore unidentified costs. Our study suggests one potential cost. Although previous host-adaptation studies sometimes demonstrated that specialists and generalists evolved to utilize hosts equally, we are unaware of any prior study that seeks to identify an ecological factor where host generalists are disadvantaged. We found that adaptive generalization incurred a performance cost for VSV at extreme temperatures. Although one might argue that this niche-space defining a cost to generalization is rather narrow, the point is that it exists and can be measured despite standing genetic divergence within and among specialists and generalists. We therefore encourage other researchers to similarly examine whether evolved host generalization is costly in unselected habitats.

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