

Incongruent fitness landscapes, not tradeoffs, dominate the adaptation of vesicular stomatitis virus to novel host types

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Host radiation refers to the ability of parasites to adapt to new environments and expand or change their niches. Adaptation to one specific environment may involve a loss in adaptation to a second environment. Thus, fitness costs may impose limits to niche expansion and constitute the cost of specialization. Several reports have addressed the cost of host radiation in vesicular stomatitis virus (VSV), but in some cases the experimental setup may have resulted in the overestimation of fitness costs. To clarify this issue, experiments were carried out in which a reference strain of VSV was allowed to adapt to HeLa, MDCK and BHK-21 cells, and to a regime of alternation between HeLa and Madin–Darby canine kidney (MDCK) cells. Measurement of viral fitness on each cell type showed that most virus populations behaved as generalists, and increased in fitness in all environments. Tradeoffs, where a fitness increase in one environment led to a fitness decrease in another environment, were rare. These results highlight the importance of using appropriate methods to measure fitness in evolved virus populations, and provide further support to a model of evolutionary dynamics in which costs due to incongruent landscapes provided by different environments are more common than tradeoffs.

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INTRODUCTION

Environmental heterogeneity contributes to the evolution of generalists and specialists. The availability of multiple niches reduces competition for resources and expands genetic diversity (Kassen & Rainey, 2004). As a population replicates, variation arises through mutation and recombination, and the fate of this variation depends on the selective pressure and random drift. In homogeneous environments, variants within populations can co-exist for a time, but higher fitness mutants will increase in frequency and replace less fit mutants (Clarke *et al.*, 1994). Adaptation to a homogeneous environment may occur at a price for survival in other environments. This cost may promote radiation and eventually lead to speciation (Kassen, 2002).

When a population replicates in heterogeneous environments, several different outcomes are possible. Firstly, a population may become adapted to a specific niche within that environment. In this case, the results are similar to those in homogeneous environments. Secondly, a population may replicate across the heterogeneous environment and adapt to all the niches within the environment, rather than adapting to a single niche. In this case, the population may experience cost of generalization, i.e. suboptimal adaptation to each niche within the environment. The

population is able to survive in each niche, but cannot reach the fitness level achieved by a specialist for that niche (Kassen, 2002). The level of variation amongst niches and the amount of time specific niches are available can influence the level of adaptation (Whitlock, 1992).

The cost of specialization can have two sources: differences in the shape of fitness landscapes, which we will refer to as ‘incongruent fitness landscapes’, and tradeoffs. Fitness is defined as the overall replicative ability of a population, and fitness landscapes represent the fitness value of different genotypes (and phenotypes), in which peaks are occupied by high-fitness genotypes whilst valleys are populated by low-fitness phenotypes. In the case of incongruent fitness landscapes, a specialized population may increase in fitness in an alternative environment as it adapts to its own environment, but not as much as a population that adapts to and specializes in that alternative environment. Thus, if the two populations were to compete, the specialist would win (Buckling *et al.*, 2003). The second source of costs is tradeoffs, where replication in one environment results in loss of fitness in an alternative environment (Elena & Lenski, 2003).

Many pathogens cycle between hosts. In particular, arboviruses (arthropod-borne viruses) undergo frequent

and potentially dramatic changes in environment, as they alternate between arthropod and vertebrate hosts. A frequently used model system to study arbovirus evolution is vesicular stomatitis virus (VSV) (Novella, 2003). VSV is a non-segmented, negative-strand RNA virus that belongs to the family *Rhabdoviridae*. Its short replication time, wide host range and high mutation rate make it a valuable tool in the study of ecology and evolution (Domingo *et al.*, 2001). The first comparison of fitness evolution during arbovirus replication in homogeneous or temporally heterogeneous niches described the behaviour of VSV replicating in mammalian and/or insect cell lines (Novella *et al.*, 1999). This report found no tradeoffs associated with differences in host-cell types, but some cost associated with differences in the temperature at which each cell line grows (Novella *et al.*, 1999). Later, Turner & Elena (2000) reported that adapting VSV to HeLa and Madin–Darby canine kidney (MDCK) cells resulted in increased fitness in the selective environment, but caused fitness tradeoffs in the other cell lines. Remold *et al.* (2008) attributed these fitness tradeoffs to mutation accumulation in the case of MDCK cell-adapted virus and to antagonistic pleiotropy for HeLa cell-adapted virus.

Here, we revisit the experimental setting of Turner & Elena (2000), because two aspects of their experiments may have resulted in spurious tradeoffs. Firstly, the sequences of the different genotypes showed polymorphisms [e.g. a mixture of wild-type (wt) and mutant nucleotides] at the site of the genetic marker used to distinguish competitors in fitness determinations (Remold *et al.*, 2008). Secondly, fitness was measured against different reference strains in different cell lines (Turner & Elena, 2000). Here, we subjected the same clone of VSV (MARM U) to serial passages on two novel hosts (MDCK and HeLa cell lines) and to a regime of alternation between the two novel hosts, under conditions that promote adaptation. We competed the adapted virus strains against the same wt strain on both cell lines and on the ancestral host (BHK-21 cells), and verified that our fitness measurements were not confounded by variability at the marker site. We found that fitness tradeoffs were rare. Only some of the populations adapted to HeLa cells showed signs of a tradeoff on the other cell lines. Populations adapted to MDCK cells, to BHK-21 cells or to fluctuating environments showed no tradeoffs. However, landscape incongruities were common. Almost all populations had a different fitness in different environments.

RESULTS

Conceptual framework

We are interested in the general scenario where an RNA virus is adapted to one environment (which we refer to as the ‘selective environment’) and then its fitness is measured in both the selective environment and in a second, ‘other’ environment. Fitness is measured through competition experiments with wt. Here, we use the term wt to refer to

the virus before it was exposed to the selective environment. Before adaptation, the virus has the same relative fitness (1.0) in both the selective and the other environment, as confirmed experimentally.

After adaptation, fitness will generally have increased in the selective environment. If we now consider the same population in the other environment, there are four possibilities (Fig. 1). Firstly, fitness may be somewhat lower in the other environment than in the selective environment but higher than before adaptation. Secondly, fitness in the other environment may be lower than before adaptation. Thirdly, fitness may be exactly the same in both environments. This outcome is possible but unlikely. And fourthly, fitness may actually be higher in the other environment than in the selective environment. This outcome is also not particularly likely, but it could occur.

Under possibilities one and two, the virus has incurred fitness costs in the other environment from adapting to the selective environment, whereas under possibilities three and four, the virus has not incurred any such fitness costs. Possibility two is a tradeoff scenario. Adaptation to one environment actually causes the virus to perform worse in the other environment. Possibility one, on the other hand,

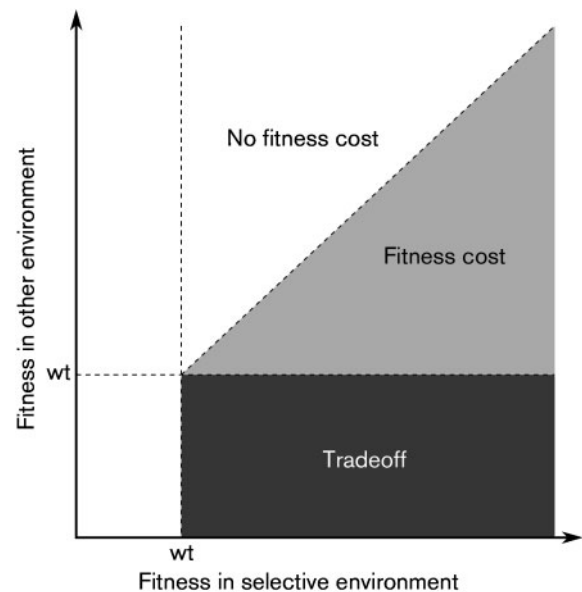


Fig. 1. Potential outcomes of adaptation. Fitness of an adapted virus relative to wt is measured in both the selective and in another environment. If fitness is higher in the selective environment, the adaptation results in fitness costs in the other environment. We subdivide these fitness costs into two scenarios. If adaptation to the selective environment actually causes fitness loss in the other environment, we say that the virus has experienced tradeoff. Alternatively, we speak of incongruent fitness landscapes: because the two landscapes are not exactly the same, fitness in the selective environment will often be somewhat higher than fitness in the other environment.

does not correspond to a tradeoff scenario because the virus improves in both environments. We refer to possibility one as ‘incongruent fitness landscapes’. Under this possibility, we observe fitness differences because the fitness landscapes in the two environments are not exactly the same.

Strictly speaking, we could speak of fitness-landscape incongruities even under the fourth scenario, when fitness is higher in the other environment than in the selective environment. But we believe that it is clearer to refer to this scenario as ‘absence of fitness costs in the other environment’.

Testing for I1 antibody sensitivity and frequency-dependent selection

We adapted MARM U to several new host environments, as described in Methods. After adaptation, we carried out sensitivity assays to determine whether the adapted populations were polymorphic at the nucleotide that confers antibody resistance. We compared plaque numbers in the presence and absence of I1 monoclonal antibody (mAb) separately for all adapted populations. We found no evidence for I1 mAb sensitivity (Table 1). Thus, we concluded that competition experiments between evolved populations, marked populations and unmarked wt would properly assess viral fitness in our experiments.

To avoid the problem of accurate wt frequency when there was MARM U excess after competition, we carried out fitness assays with an excess of wt, i.e. with a ratio of MARM U to wt different from 1 : 1, such as 1 : 10 or 1 : 100. With an initial excess ratio, the wt frequency will still be sufficiently high after competition and the fitness can be determined accurately. One potential problem with this

approach is that it can produce meaningless results if selection is frequency dependent. Frequency-dependent selection is uncommon but possible in regimes of acute infection in VSV (Elena *et al.*, 1997; Wilke *et al.*, 2004). To test whether frequency-dependent selection would confound our results, we carried out competitions at different initial ratios of MARM U:wt and looked for changes in fitness as the ratio of wt to MARM changed. Our results showed no correlation between the measured fitness and the initial ratio of MARM U:wt (Table 2). Therefore, we concluded that fitness competitions carried out with an excess of wt were meaningful for our system.

Assessing incongruent fitness landscapes and tradeoffs

Passaging MARM U on a host for 25 passages allowed the virus to adapt to that environment (Table 3 and Figs 2 and 3). HeLa cell-adapted virus increased its fitness in HeLa cells, MDCK cell-adapted virus increased its fitness in MDCK cells, and BHK-21 cell-adapted virus increased its fitness in BHK-21 cells; these results are consistent with previous work (Novella *et al.*, 1995b, 2004). Alternating environments between MDCK and HeLa cells led to a significant increase in viral fitness on both cell lines at passages 25 and 50, as predicted by theory and in accordance with previous work (Table 3) (Novella *et al.*, 1999; Weaver *et al.*, 1999). Between passage 25 and passage 50 of virus cycling between MDCK and HeLa cells, there was no measurable increase in fitness of that virus on either cell line (Table 4, bottom row).

As expected, the host cell type where we tested each evolved strain had an effect on the fitness of the strain, but we found no consistent pattern of tradeoffs, in contrast to a previous report (Turner & Elena, 2000). Virus adapted to HeLa cells did not systematically gain or lose fitness on either MDCK or BHK-21 cells (Table 3). There was significant variation among replicates when competed on MDCK cells, as seen by the non-zero random intercepts we obtained (Table 3); replicates C and D experienced significant fitness loss, whilst replicates A and B did not (Table 5). In all other cases (virus adapted to MDCK cells, to BHK-21 cells or to cycling between MDCK and HeLa), the virus gained fitness on all three cell types (Table 3).

Variation among replicates was common. In more than half of all conditions, fitness among replicates differed significantly (last two columns of Table 3). Interestingly, fitness variability seemed to depend to some extent on the cell type on which virus populations were competed. Fitness was consistently more variable on MDCK cells than on either HeLa or BHK-21 cells. The latter two were roughly equal in the amount of variability they produced.

We also calculated the fitness differences of all adapted populations on different cell types. Fitness on HeLa cells was consistently higher than fitness on BHK-21 cells, regardless of the conditions under which populations were

Table 1. Test for I1 mAb sensitivity

Adapted to:	Replica	Passage	Plaque count*		P value (t-test)
			Total	I1	
HeLa	A	25	228 ± 3.4	226 ± 10.5	0.91
	B	25	175 ± 10.1	167 ± 6.8	0.55
	C	25	197 ± 7.4	205 ± 3.9	0.41
	D	25	158 ± 5.9	162 ± 4.8	0.65
MDCK	A	25	157 ± 4.7	154 ± 6.8	0.74
	B	25	164 ± 5.8	161 ± 4.6	0.74
	C	25	244 ± 4.5	236 ± 3.2	0.24
	D	25	203 ± 5.7	209 ± 5.3	0.46
M/H†	A	50	201 ± 9.0	206 ± 4.2	0.69
	B	50	166 ± 5.8	157 ± 12.8	0.56
	C	50	248 ± 9.3	256 ± 10.8	0.59
	D	50	213 ± 27.4	202 ± 13.7	0.74

*Mean ± SEM.

†Alternation between MDCK and HeLa host cells.

Table 2. Test for frequency dependence

Adapted to:	Strain	Competed on:	wt excess (log fitness*)			P value (linear regression)
HeLa	C25	HeLa	5 (3.13 ± 0.12)	10 (2.86 ± 0.36)	20 (2.99 ± 0.20)	0.777
		MDCK	1 (-1.82 ± 0.30)	5 (-1.74 ± 0.08)	20 (-1.85 ± 0.19)	0.776
MDCK	B25	HeLa	5 (2.87 ± 0.16)	10 (2.70 ± 0.18)	20 (2.69 ± 0.11)	0.402
		MDCK	10 (3.44 ± 0.29)	20 (3.68 ± 0.09)	40 (3.60 ± 0.39)	0.702
M/H†	B50	HeLa	10 (2.99 ± 0.60)	20 (2.63 ± 0.25)	40 (2.98 ± 0.28)	0.911
		MDCK	10 (2.83 ± 0.20)	20 (3.46 ± 0.32)	40 (2.99 ± 0.30)	0.931

*Log-transformed fitness ± SEM.

†M/H, Alternation between MDCK and HeLa host cells.

adapted (Table 4). We found no similar relationship for the other two pairings. Fitness on HeLa cells was higher than fitness on MDCK cells for populations adapted to HeLa and BHK-21 cells, but not for populations adapted to MDCK cells or to alternating regimes of MDCK and HeLa cells. Fitness on BHK-21 cells was higher than fitness on MDCK cells for populations adapted to HeLa cells, but was lower than fitness on MDCK cells for populations adapted to MDCK or alternating regimes, and was virtually identical to the fitness on MDCK cells for populations adapted to BHK-21 cells (Table 4).

The majority of the evolved populations in our study showed no fitness tradeoffs, but we identified widespread incongruities among fitness landscapes. With the exception of two cases, adapted populations had a different fitness on

different cell types (Table 4). At the same time, most strains behaved as generalists rather than as specialists. Fitness increases under one condition generally also led to fitness increases under a different condition.

DISCUSSION

Using the arbovirus VSV as a model system, we asked whether adaptation to one environment comes at a fitness cost in other environments. Our results indicated that such fitness tradeoffs are rare in VSV adapted under laboratory conditions. We found only one replica adapted to HeLa cells that experienced a tradeoff in MDCK and BHK-21 cells, and a second one that experienced a tradeoff in MDCK but not BHK-21 cells. All other replicas showed no

Table 3. Joint mixed-model analysis of competition results for all four replicas adapted to one cell type and competed on one cell type

Adapted to:	Competed on:	Mean log fitness*	P value	SD of random intercepts†	P value
HeLa	HeLa	3.34 ± 0.47	<10 ⁻¹⁰ ‡	0.90	0.015‡
	MDCK	-1.15 ± 0.61	0.059	1.16	0.005‡
	BHK-21	0.13 ± 0.15	0.380	0.21	0.224
MDCK	HeLa	2.53 ± 0.28	<10 ⁻¹⁰ ‡	0.43	0.123
	MDCK	2.97 ± 0.42	<10 ⁻¹⁰ ‡	0.76	0.029‡
	BHK-21	0.78 ± 0.18	<0.0001‡	0.35	0.005‡
BHK-21	HeLa	1.97 ± 0.23	<10 ⁻¹⁰ ‡	0.34	0.180
	MDCK	1.05 ± 0.40	0.008‡	0.74	0.030‡
	BHK-21	1.04 ± 0.20	<0.0001‡	0.37	0.009‡
M/H25§	HeLa	3.09 ± 0.15	<10 ⁻¹⁰ ‡	0.00	1.000
	MDCK	4.15 ± 0.59	<10 ⁻¹⁰ ‡	1.10	0.031‡
	BHK-21	0.74 ± 0.22	<0.001‡	0.41	0.038‡
M/H50§	HeLa	3.19 ± 0.33	<10 ⁻¹⁰ ‡	0.00	1.000
	MDCK	3.83 ± 0.39	<10 ⁻¹⁰ ‡	0.62	0.111
	BHK-21	0.86 ± 0.16	<0.0001‡	0.00	1.000

*Estimated mean log fitness ± SEM of all four replicates in comparison with wt.

†The statistical model allows each individual replicate to deviate in its log fitness from the estimated mean. The SD of random intercepts estimates the magnitude of these deviations.

‡Statistically significant at $\alpha=0.05$.

§M/H25 and M/H50, passages 25 and 50, respectively, of alternation between MDCK and HeLa host cells.

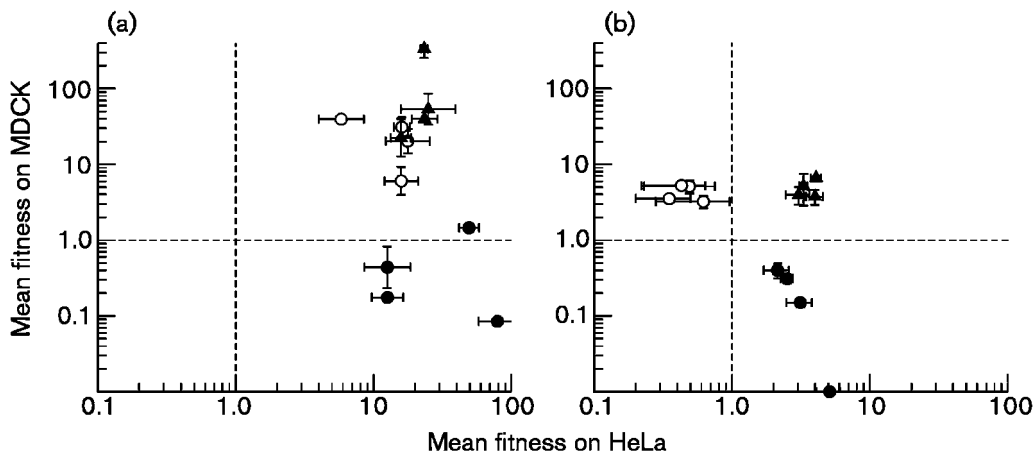


Fig. 2. Fitness changes during adaptation of VSV to novel host cells (MDCK and HeLa). (a) Fitness on HeLa cells versus fitness on MDCK cells for virus adapted to HeLa cells (●), MDCK cells (○) or a regime of alternation between HeLa and MDCK cells (▲). (b) Results of Turner & Elena (2000) for the same experiment. Symbols are the same as in (a).

evidence of tradeoffs. Instead, we found abundant evidence for fitness landscape incongruity. Fitness on one cell type generally differed from fitness on another cell type, but there was no systematic pattern to the magnitude and sign of this difference. Thus, both incongruent landscapes and tradeoffs are possible and, whilst the latter are rare, they may be affected by small differences in the experimental conditions. For instance, fitness measurements of *Escherichia coli* populations varied by about 10% due to different water sources (O’Keefe *et al.*, 2006).

In this study, we showed that HeLa cell-adapted virus did not lose fitness on BHK-21 cells, but rather failed to increase in fitness, illustrating a cost associated with

incongruent fitness landscapes. Other studies using cell culture to analyse the evolution of other RNA viruses have produced evidence of cell-specific adaptation as well as evidence for adaptation across environments. Tradeoffs during adaptation to insect and mammalian cells have been reported in Sindbis virus (Greene *et al.*, 2005), dengue virus (Chen *et al.*, 2003), eastern equine encephalitis (Weaver *et al.*, 1999; Cooper & Scott, 2001) and Venezuelan equine encephalitis virus (Coffey *et al.*, 2008), whilst experiments with VSV showed no tradeoffs in fitness between mammalian and invertebrate cell lines (Novella *et al.*, 1999) except during persistence (Zarate & Novella, 2004). In contrast, the cost associated with differences in landscape and the mechanism(s) of such cost are more

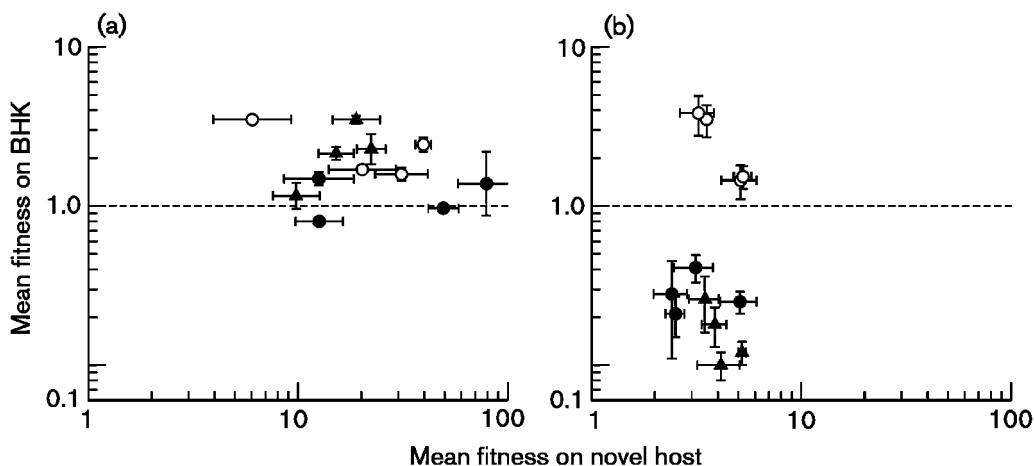


Fig. 3. Fitness changes in the original host cells (BHK-21) during adaptation of VSV to novel host cells. (a) Fitness on BHK-21 cells versus fitness on the novel host for virus adapted to HeLa cells (●), MDCK cells (○) or a regime of alternation between HeLa and MDCK cells (▲). For the latter, fitness is the geometric mean across novel cell types. (b) Results of Turner & Elena (2000) for the same experiment. Symbols are the same as in (a).

Table 4. Fitness differences among cell types on which competitions were carried out

Differences and associated *P* values correspond to Tukey's all-pairwise comparisons, calculated using the R package multcomp.

Adapted to:	Null hypothesis*	Estimate†	<i>P</i> value
HeLa	BHK-21–HeLa=0	-3.29 ± 0.40	<0.001‡
	MDCK–HeLa=0	-4.49 ± 0.38	<0.001‡
	MDCK–BHK-21=0	-1.20 ± 0.38	0.005‡
MDCK	BHK-21–HeLa=0	-1.75 ± 0.32	<0.0001‡
	MDCK–HeLa=0	0.42 ± 0.31	0.36
	MDCK–BHK-21=0	2.17 ± 0.32	<0.0001‡
BHK-21	BHK-21–HeLa=0	-0.96 ± 0.19	<0.0001‡
	MDCK–HeLa=0	-0.90 ± 0.20	<0.0001‡
	MDCK–BHK-21=0	0.06 ± 0.19	0.94
M/H§	BHK-21–HeLa=0	-2.33 ± 0.25	<0.0001‡
	MDCK–HeLa=0	0.83 ± 0.26	0.005‡
	MDCK–BHK-21=0	3.16 ± 0.25	<0.0001‡
	P50–P25=0	-0.02 ± 0.21	1.00

*Names of cell types refer to virus log fitness on that cell type. Thus, the null hypothesis 'BHK-21–HeLa=0' implies that the virus has the same fitness on BHK-21 and HeLa cells.

†Estimate (\pm SEM) of the difference in log fitness referred to in the null hypothesis.

‡Statistically significant at $\alpha=0.05$.

§M/H, Alternation between MDCK and HeLa host cells.

||P25 and P50 refer to passages 25 and 50, respectively. Rejection of this null hypothesis would indicate that fitness has changed significantly between the two time points.

difficult to predict. Studies with West Nile virus and St Louis equine encephalitis virus showed a tradeoff between invertebrate-adapted virus in mammalian cells and what appears to be a cost associated with landscapes when the invertebrate-adapted virus was tested in avian cells (Ciota *et al.*, 2007). The same studies also served to test the cost of generalization. This cost was infrequent and only clear in Sindbis virus (Greene *et al.*, 2005) and perhaps eastern equine encephalitis virus (Cooper & Scott, 2001). Research with arboviruses has shown that adaptation to homogeneous and temporally heterogeneous environments results in populations with similar fitness values (Novella *et al.*, 1999; Weaver *et al.*, 1999). Interestingly, these same *in vitro* results were not seen *in vivo* when eastern equine encephalitis virus was passaged in mice and mosquitoes; the generalist in this experiment did not gain fitness on either vertebrate or invertebrate cells (Coffey *et al.*, 2008).

Comparisons of previous results (Turner & Elena, 2000; Remold *et al.*, 2008) with those reported here allowed us to identify the causes of the discrepancies between the two studies. Both pre-adaptation of wt and the loss of marker in some of the populations will probably have altered the results. Among the evolved populations analysed previously (Turner & Elena, 2000), those adapted to HeLa cells

seemed to have retained their genetic marker (Remold *et al.*, 2008). Using pre-adapted wt for the analysis of these HeLa cell-adapted strains may have led to overall underestimation of fitness in all environments, and to the identification of tradeoffs in BHK-21 cells. Populations adapted to heterogeneous environments for 25 passages were partially sensitive to I1 mAb (Remold *et al.*, 2008). In this case, there was also an overall underestimation of fitness values, but to a much larger degree. In addition, instead of the substantial fitness costs previously reported, in the present study we found no costs during competition of alternating viruses in BHK-21 cells. Because the competitions in BHK-21 cells were carried out in both laboratories using standard wt, the differences can be assigned to the loss of genetic marker.

This study highlights some of the problems that may arise during fitness analysis of VSV in the laboratory, and here we propose means to address these problems. Marker loss can be avoided by periodic addition of I1 mAb during the experimental evolution of MARM U populations (Clarke *et al.*, 1993). This method has the disadvantage that potential effects of this site on evolution would be missed. For the marker at nt 3853, this is an unlikely problem because it has been shown to be neutral in a large number of environments (Novella *et al.*, 1995b, c; Quer *et al.*, 1996; Novella, 2004). However, resistance to I1 mAb caused by substitutions in other loci, such as at nt 3846, is frequently observed after adaptation to different environments – even though it is always in combination with a substitution at nt 2151 (Cuevas *et al.*, 2002; Novella, 2004; Remold *et al.*, 2008). Thus, the substitution at nt 3846 may sometimes have a beneficial effect and should be avoided as a genetic marker. We have found that populations evolving in BHK-21 cells always remain fully resistant to I1 mAb, even after hundreds of generations (unpublished results), but in other environments, sensitivity to I1 mAb should be carefully assessed before analysis. If partial sensitivity develops, genetic analysis would still be useful, but fitness analyses should not be performed. An alternative option is to carry out the experimental evolution with wt, which should not develop I1 mAb resistance in the absence of selective pressure, and to use MARM U as a reference.

Regarding the analysis of very high-fitness MARM U populations, there is no reason to do the competitions at an initial 1:1 ratio. Changing initial ratios and adding an excess of wt in the competition mixture is better than using a pre-adapted reference wt because there is no dependence on fitness being transitive. The only caveat is the potential existence of frequency-dependent selection; however, this is infrequent in experimental regimes where the passages are carried out at a low m.o.i. In any case, tests for frequency-dependent selection should be carried out to verify that the results are reliable. If these tests reveal frequency dependence, then the entire approach of assigning a single fitness value to each population becomes meaningless. Thus, in this case, there is no specific benefit to measuring fitness at an initial 1:1 ratio either.

Table 5. Fitness of evolved strains on various cell types and comparison with MARM U

Adapted to:	Tested on:	Replica	Passage	Fitness*	log fitness*	<i>t</i> -test against MARM U log fitness	
						<i>t</i> value	<i>P</i> value
HeLa	HeLa	A	25	13.53 ± 4.97	2.53 ± 0.39	6.40	0.041*‡
		B	25	50.00 ± 8.30	3.90 ± 0.17	20.39	0.003*‡
		C	25	13.05 ± 3.35	2.53 ± 0.26	9.12	0.021*‡
		D	25	86.93 ± 26.0	4.37 ± 0.31	13.44	0.001*‡
	MDCK	A	25	0.62 ± 0.33	-0.83 ± 0.63	-1.23	0.338
		B	25	1.48 ± 0.21	0.38 ± 0.13	2.47	0.063
		C	25	0.18 ± 0.01	-1.74 ± 0.08	-13.09	<0.0001*
		D	25	0.09 ± 0.01	-2.47 ± 0.06	-21.02	<0.0001*
	BHK-21	A	25	1.50 ± 0.15	0.40 ± 0.10	3.40	0.046*
		B	25	0.97 ± 0.03	-0.03 ± 0.03	-1.12	0.308
		C	25	0.80 ± 0.03	-0.22 ± 0.04	-4.39	0.010*
		D	25	1.54 ± 0.67	0.32 ± 0.46	0.63	0.639
MDCK	HeLa	A	25	16.50 ± 4.50	2.76 ± 0.28	9.40	0.043*
		B	25	16.15 ± 2.15	2.77 ± 0.13	17.06	0.003*
		C	25	6.60 ± 2.04	1.76 ± 0.38	4.56	0.036*
		D	25	19.97 ± 6.13	2.87 ± 0.37	7.59	0.012*
	MDCK	A	25	7.37 ± 3.42	1.80 ± 0.43	4.17	0.022*‡
		B	25	33.63 ± 8.87	3.44 ± 0.29	11.39	0.002*‡
		C	25	39.75 ± 3.45	3.68 ± 0.09	28.11	<0.0001*‡
		D	25	23.40 ± 9.33	3.01 ± 0.37	7.98	0.005*‡
	BHK-21	A	25	3.50 ± 0.20	1.25 ± 0.06	16.97	0.002*
		B	25	1.60 ± 0.15	0.46 ± 0.09	4.22	0.025*
		C	25	2.45 ± 0.25	0.89 ± 0.10	7.74	0.041*
		D	25	1.70 ± 0.10	0.53 ± 0.06	6.81	0.015*
BHK-21	HeLa	A	25	6.00 ± 2.42	1.64 ± 0.38	4.17	0.044*
		B	25	5.13 ± 1.16	1.58 ± 0.23	6.30	0.012*
		C	25	11.20 ± 0.40	2.42 ± 0.04	24.38	<0.0001*
		D	25	11.10 ± 2.40	2.38 ± 0.22	10.02	0.030*
	MDCK	A	25	3.40 ± 0.72	1.18 ± 0.22	5.04	0.017*
		B	25	0.90 ± 0.20	-0.13 ± 0.23	-0.38	0.751
		C	25	6.37 ± 2.05	1.71 ± 0.41	4.18	0.043*
		D	25	4.10 ± 0.10	1.41 ± 0.02	14.12	<0.0001*
	BHK-21	A	25	1.93 ± 0.38	0.62 ± 0.19	3.11	0.039*‡
		B	25	2.37 ± 0.50	0.82 ± 0.21	3.68	0.029*‡
		C	25	4.68 ± 0.61	1.52 ± 0.13	11.02	0.0003*‡
		D	25	3.23 ± 0.20	1.17 ± 0.06	14.95	<0.0001*‡
M/H	HeLa	A	25	23.35 ± 1.35	3.15 ± 0.06	28.88	<0.0001*
		B	25	23.95 ± 5.05	3.15 ± 0.21	13.54	0.019*
		C	25	29.80 ± 10.9	3.22 ± 0.45	6.94	0.016*
		D	25	16.00 ± 2.70	2.76 ± 0.17	14.25	0.010*
		A	50	15.10 ± 4.00	2.68 ± 0.27	9.36	0.042*
		B	50	29.03 ± 17.6	2.99 ± 0.60	4.91	0.036*
		C	50	19.05 ± 0.05	2.95 ± 0.003	31.86	<0.0001*
		D	50	88.07 ± 42.5	3.90 ± 0.94	4.15	0.052
	MDCK	A	25	351.50 ± 98.5	5.82 ± 0.29	19.23	0.017*
		B	25	40.00 ± 0.00	3.69 ± 0.00	37.44	<0.0001*
		C	25	59.65 ± 26.4	3.98 ± 0.47	8.29	0.064
		D	25	26.15 ± 13.5	3.11 ± 0.57	5.45	0.105
		A	50	158.33 ± 56.3	4.95 ± 0.33	14.34	0.002*
		B	50	35.63 ± 12.3	3.46 ± 0.32	10.41	0.005*
		C	50	33.10 ± 14.4	3.39 ± 0.47	7.20	0.075
		D	50	47.43 ± 29.6	3.43 ± 0.66	5.20	0.032*
	BHK-21	A	25	1.18 ± 0.22	0.15 ± 0.19	0.61	0.642
		B	25	2.40 ± 0.56	0.83 ± 0.22	3.57	0.063

Table 5. cont.

Adapted to:	Tested on:	Replica	Passage	Fitness*	log fitness*	t-test against MARM U log fitness	
						t value	P value
M/H	BHK-21	C	25	2.13 ± 0.20	0.75 ± 0.09	6.89	0.007*
		D	25	3.50 ± 0.20	1.25 ± 0.06	16.97	0.002*
		A	50	3.03 ± 0.90	1.00 ± 0.35	2.78	0.105
		B	50	2.63 ± 1.13	0.80 ± 0.40	1.93	0.191
		C	50	3.13 ± 0.49	1.12 ± 0.16	6.56	0.015*
		D	50	1.97 ± 0.74	0.53 ± 0.38	1.29	0.323

*Mean fitness or mean log-transformed fitness ± SEM.

†Statistically significant at $\alpha=0.05$.

‡We calculated one-sided *P* values whenever we measured fitness on the cell type to which a strain was adapted, because we always expect fitness to increase compared with wt in this scenario.

Previous articles have pointed out that perhaps fitness tradeoffs are overrated as the driving cause of specialization (Fry, 1996), and have asked whether fitness costs due to differences in landscape profiles are too often overlooked. Experimental work with *Drosophila* (Weber, 1996; Bolnick, 2001), bacteria (Rainey & Travisano, 1998; Sander *et al.*, 2002; Buckling *et al.*, 2003; MacLean *et al.*, 2004) and viruses (Novella *et al.*, 1995a; Feuer *et al.*, 1999; Cooper & Scott, 2001; Zarate & Novella, 2004; Ciota *et al.*, 2007), as well as observations of natural occurrences of diverged species (Schliewen *et al.*, 2001; McKinnon & Rundle, 2002), have led to the identification of tradeoffs as the potential cause of speciation, yet very few works have specifically addressed costs due to landscape differences, or they fail to correctly identify them, leaving a void in our knowledge about which mechanism contributes more to speciation. In this regard, our results suggest that incongruous fitness landscapes may have a more important role than tradeoffs in the evolution of some arboviruses.

In conclusion, the results from this study and others show that costs due to differences in landscapes are more common than costs due to tradeoffs, and the contributions of the former to radiation, and potentially to speciation, may be more significant than currently thought.

METHODS

The virus strains, cell lines and methods used were identical to those described by Turner & Elena (2000), unless indicated otherwise.

Viruses and cells. We used two genotypes of VSV Indiana serotype, Mudd-Summers strain: I1 mAb-sensitive wt and MARM U, an I1 mAb-resistant clone. Remold *et al.* (2008) reported that MARM C was the progenitor of MARM U; despite the difference in label between laboratories, MARM U and MARM C are the same clone, which Holland *et al.* (1991) isolated, characterized and provided to different groups. We also used BHK-21 cells for experimental passages and plaque assays. HeLa and MDCK cells were from the European Collection of Cell Cultures.

Experimental passages. Briefly, MARM U was used to initiate four replicas each (labelled A–D) of four experimental regimes: 25 passages

in either MDCK or HeLa cells, which served as novel cell hosts; 25 passages in BHK-21 cells, which are typically used to propagate VSV; and alternating between MDCK and HeLa for 25 passages. In our experimental design, we continued alternating passages for an additional 25 passages (for a total of 50 alternating passages, 25 on each cell type). Infections were allowed to proceed for 24 h (BHK-21 cell infections) or 48 h (MDCK and HeLa cell infections) at 37 °C and the progeny were diluted 10^{-4} to carry out the next passage. The final passages were collected and stored at -80 °C for further analysis. All populations were tested for I1 mAb sensitivity to determine whether they had retained the phenotypic marker used for fitness determinations. We assayed sensitivity by triplicate plaque assays in the presence and absence of I1mAb; plaques were counted and mean values were compared using *t*-tests.

Fitness determinations. Fitness assays were carried out as described previously, by direct competition between I1 mAb-resistant, evolved virus and I1 mAb-sensitive reference wt (Holland *et al.*, 1991) in HeLa, MDCK, and BHK-21 cells. We measured relative MARM U:wt ratios by triplicate plaque assays in the presence and absence of I1 mAb and followed the changes in ratio before (R0) and after (R1) competition. Fitness was calculated as R1/R0, and each fitness value was the mean of at least two independent determinations. We confirmed the neutrality of the genetic marker in each of the cell lines by carrying out seven competitions between wt and MARM U. Fitness values (\pm SEM), with log fitness in parentheses, were 1.04 ± 0.05 (0.029 ± 0.04) in BHK-21 cells, 0.99 ± 0.10 (-0.036 ± 0.10) in MDCK cells and 1.02 ± 0.09 (-0.006 ± 0.09) in HeLa cells.

When we let adapted, high-fitness MARM U strains compete with wt at initial ratios of 1:1, we found that we could not obtain accurate fitness measurements (not shown). This issue had previously been encountered by Turner & Elena (2000). The problem originates from the way that fitness is measured: we determined ratios by counting plaque numbers produced in the presence and absence of I1 mAb, and then compared the ratio of MARM:wt before and after competition. Counts in the presence of I1 mAb gave the number of MARM U virus particles, and in the absence of I1 both wt and MARM U produced plaques. We then calculated the amount of wt as the difference between total virus (produced in the absence of I1 mAb) and MARM U virus (produced in the presence of I1). Thus, when MARM U was in substantial excess, the plaque numbers with and without I1 mAb were similar, and the fraction that represented wt could not be calculated with any accuracy. For counts of about 150–250 plaques, our cutoff was a minimum of 10% wt for reliable measures. Therefore, the high fitness achieved by the adapted virus

exceeded the detection limit of the fitness assay when we started the competitions at a 1:1 ratio.

Turner & Elena (2000) circumvented this problem by pre-adapting wt reference virus independently to the two new environments (HeLa and MCDK cells). They then did fitness competitions against three different reference strains: parental wt, HeLa cell pre-adapted wt and MDCK cell pre-adapted wt. Because pre-adapted wt had increased fitness, it could be detected after competition against evolved MARM U genotypes. The drawback of this approach is that it assumes that fitness is transitive, i.e. the fitness of evolved MARM U against parental wt has to be the same as the fitness of evolved MARM U against pre-adapted wt times the fitness of pre-adapted wt against parental wt.

Our approach to address the problem of inaccuracy was different. We first competed selected populations from each regime at initial ratios of between 1:1 and 40:1 and showed that there was no frequency-dependent selection (Table 2). We then completed the fitness assays by adjusting the initial MARM U:wt ratio as needed.

Statistical analyses. All statistical analyses were carried out with the software platform R (<http://www.r-project.org/>). We used the package lme4 (<http://lme4.r-forge.r-project.org>) to fit mixed linear models, and used the packages multcomp (Hothorn *et al.*, 2008) and RLRsim (Scheipl *et al.*, 2008) to test for the significance of fixed and random effects, respectively. Unless stated otherwise, all analyses of virus fitness employed a linear mixed model with a single fixed-effect term representing the mean fitness of all replicates evolved under and competed on a single condition and a random-effects term representing variability among replicates. All fitness values were log transformed before statistical analysis. We used the natural log (ln) in this transformation.

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