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Author for correspondence:
Valerie J. Morley
e-mail: valerie.morley@yale.edu

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Rate of novel host invasion affects adaptability of evolving RNA virus lineages

Valerie J. Morley, Sandra Y. Mendiola and Paul E. Turner

Department of Ecology and Evolutionary Biology, Yale University, PO Box 208106, 165 Prospect Street, New Haven, CT 06520-8106, USA

Although differing rates of environmental turnover should be consequential for the dynamics of adaptive change, this idea has been rarely examined outside of theory. In particular, the importance of RNA viruses in disease emergence warrants experiments testing how differing rates of novel host invasion may impact the ability of viruses to adaptively shift onto a novel host. To test whether the rate of environmental turnover influences adaptation, we experimentally evolved 144 Sindbis virus lineages in replicated tissue-culture environments, which transitioned from being dominated by a permissive host cell type to a novel host cell type. The rate at which the novel host 'invaded' the environment varied by treatment. The fitness (growth rate) of evolved virus populations was measured on each host type, and molecular substitutions were mapped via whole genome consensus sequencing. Results showed that virus populations more consistently reached high fitness levels on the novel host when the novel host 'invaded' the environment more gradually, and gradual invasion resulted in less variable genomic outcomes. Moreover, virus populations that experienced a rapid shift onto the novel host converged upon different genotypes than populations that experienced a gradual shift onto the novel host, suggesting a strong effect of historical contingency.

1. Introduction

In nature, asexual populations frequently evolve in complex and dynamic environments. When a population experiences a change in its environment, we expect it to adapt by fixing a series of beneficial mutations that improve its reproductive fitness under the new environmental conditions. An environmental change experienced by a virus population may be very sudden. If a mosquito carrying a zoonotic arthropod-borne virus rides on a logging truck from the forest into an urban centre, the virus it carries has suddenly been transplanted from a host community dominated by non-human vertebrate hosts to one dominated by humans. The dynamics of adaptation in response to a sudden environmental change, after which a population adapts to a novel but constant environment, have been well characterized [1,2]. At other times, however, environmental turnover might happen more gradually. Perhaps humans gradually encroach onto the aforementioned forest habitat, and the virus-infected forest mosquitos are increasingly likely to bite a human versus another vertebrate when foraging. In this case, the virus population experiences more gradual turnover of its host community. Understanding how the rate of environmental change influences adaptation is important if we hope to predict how populations will respond to complex environmental changes, ranging from shifts in host community composition to climate change.

This study examines populations evolving in directionally changing heterogeneous environments, where the increasingly novel environment constitutes a stressor (i.e. challenge that reduces performance, relative to the original environment). In the directionally changing scenario, a population experiences a gradual environmental change rather than a sudden one and is forced to track

a moving optimum as the stressful environment increases over time [3]. In a gradually changing environment, the population experiences a series of smaller drops in fitness and may fix a series of smaller effect mutations in response [4–6]. The rate of environmental change may affect the likelihood of extinction [7–11], fitness in the final environment [9,12] and which mutations go to fixation [4–6,9,12,13].

We examined how an environmental change of fixed magnitude but variable rate influenced evolutionary outcomes for an RNA virus. Sindbis virus (SINV) is a positive-sense single-stranded RNA virus with a non-segmented 11.7 kb genome. The genome encodes nine genes: four non-structural proteins (nsp1, nsp2, nsp3 and nsp4) and five structural proteins (C, E3, 6K, E1 and E2). SINV naturally cycles primarily between passerine birds and *Culex* mosquito species, with spillover infections causing fever and arthralgia in humans. SINV serves as a model for other positive-sense RNA viruses and is easily grown in laboratory tissue culture. In the current experiment, replicated SINV lineages experienced a shift from a permissive (original) host cell type to a challenging novel host cell type, but how quickly the novel host 'invaded' the environment varied among treatments. Evolved virus populations were assayed for fitness on both the novel host cell type and the permissive host cell type, and all evolved virus genomes were fully sequenced. Our experiment addresses three key questions. When virus populations experience a shift onto a new host type suddenly versus gradually, do the evolved populations achieve the same phenotype (fitness) in the time allowed? Do populations that experience different rates of environmental turnover converge upon similar genomic changes relative to the common ancestor, or do their genomes diverge? How does the rate of novel host invasion influence the repeatability of adaptation for independently evolving virus lineages experiencing identical environmental challenges?

We found that virus lineages more consistently reached high levels of fitness on the novel host in response to more gradual novel host invasion. Gradual invasion led to reduced phenotypic variation among evolved populations, as well as reduced genomic variation among populations within a treatment. This suggests that evolution may be more predictable both at the phenotypic and genomic levels in environments that change gradually. We also inferred that epistasis was common, which may have contributed to the constrained adaptive outcomes in populations that evolved in mixed host environments.

2. Material and methods

(a) Viruses and cell culture

(i) Sindbis virus

Virus was derived from the infectious clone pTOTO1101 [14]. To create an ancestral virus stock, the virus was transcribed and amplified for 24 h on BHK-21 cells as previously described [14]. All experimental virus lines were founded from this ancestral stock.

(ii) Host cells

SINV was cultured on two host cell lines: BHK-21 and CHO. BHK-21 is a golden hamster (*Mesocricetus auratus*) kidney derived cell line that is highly permissive for virus replication. CHO cells are derived from Chinese hamster (*Cricetulus griseus*) ovaries, and the CHO cell line used for this experiment was a genetically modified line deficient in heparin sulfate synthesis (pgsD-677 ATCC CRL-2244). CHO cells deficient in heparin sulfate are known to be more resistant to SINV infection relative to cells with unmodified heparin sulfate synthesis pathways, so these cells pose a novel challenge to evolving SINV populations despite the fact that both cell lines are derived from related host species [15]. Both cell lines were cultured in Ham's *F*-12 K (Kaighn's) medium (Life Technologies) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. For each transfer, cells were counted on a haemocytometer and seeded at approximately 1.4×10^5 cells per well in 24-well tissue-culture plates, then allowed to grow for 24 h to achieve confluence prior to virus infection. Cells were incubated at 37°C, 95% relative humidity and 5% CO_2 atmosphere.

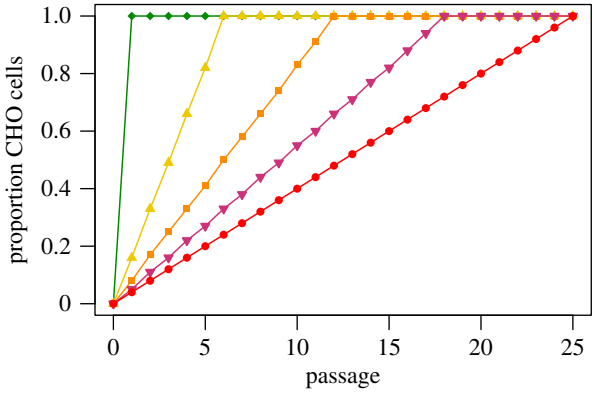

Figure 1. The five experimental treatments for serial transfer. The percentage of CHO cells (novel host) in the environment increases from 0 to 100%, but the rate of increase varies among treatments. Each line represents the passaging regime for one treatment: treatment 1 (green diamonds), treatment 2 (yellow up-triangles), treatment 3 (orange squares), treatment 4 (pink down-triangles) and treatment 5 (red circles). Symbols mark the proportion of CHO cells in the environment at each passage.

(b) Cell imaging

Each cell type was stained separately with a fluorescent dye, either CellTracker Green CMFDA Dye (Life Technologies C2925) or Orange CMFDA Dye (C2927). The stained cells were then combined to form mixed cell monolayers on glass slides placed in tissue-culture dishes. Monolayers were fixed using 4% paraformaldehyde, imaged using confocal microscopy and analysed by visual inspection.

(c) Experimental evolution of virus populations

The ancestral virus stock was used to found 24 replicate lineages in each of six treatments (144 lineages total). Over the course of the experiment, the virus populations in five experimental treatments experienced a shift to a novel host cell type (CHO), but how quickly the new host 'invaded' varied among treatments. During each passage, the evolving virus lineage experienced some combination of BHK-21 cells and CHO cells, until eventually reaching a passage consisting of solely CHO cells that remained constant for the remainder of the experiment (figure 1). In treatment 1, which represented a sudden change, viral populations were grown on 100% CHO cells starting from the first passage. In the early passages of treatment 2, populations were grown on mixed monolayers of BHK-21 and CHO cells, in which the frequency of CHO cells increased by approximately 16% per passage. By passage 6, the populations in treatment 2 were growing in cultures of 100% CHO cells. The remaining treatments contained progressively slower rates of environmental turnover, with cultures reaching 100% CHO

cells at passages 12 (treatment 3), 18 (treatment 4) and 25 (treatment 5). Additionally, 24 replicate virus populations were evolved on BHK cells for the duration of the experiment as a control (treatment 6). To initiate each treatment population, a cell monolayer was infected with the ancestral virus at a multiplicity of infection (MOI) of approximately 0.01 plaque-forming units (pfu) per cell. The infected monolayer was incubated at 37°C for 48 h. Then, a subset of each virus population (approx. 10^4 virus particles per transfer) was transferred to fresh cells. A subset of virus lineages (four per treatment) were titred every five passages to ensure that effective population sizes were similar among treatments. A different set of lineages was titred at each checkpoint. This process was repeated for 25 passages total. At each transfer, aliquots of supernatant containing the virus progeny were drawn from each lineage and frozen at −80°C for future analysis.

(d) Plaque assays

Viral titres (pfu per ml) were estimated using plaque assays, in which serially diluted samples were plated on BHK cells under F12-K medium with 10% fetal bovine serum and solidified with 1% agarose, and incubated for 36 h using the above-described conditions. After incubation, cells were fixed with 10% formaldehyde, media and agarose were removed, and plates were stained with crystal violet to visualize plaques. Each plaque was assumed to have originated from a single infecting virus.

(e) Fitness assays

Growth of the ancestral and evolved virus lineages was assayed on monolayers of BHK cells and on monolayers of CHO cells. Fitness assays were performed in three blocks, with one replicate from each evolved population per block (three total replicates per population on each cell type) and three replicates of the ancestral virus stock per block (nine total replicates on each cell type). Monolayers were infected at MOI = 0.005, and incubated for 48 h in the conditions described above. After 48 h, the supernatant containing virus progeny was frozen at −80°C for future analysis. Plaque assays were performed on thawed samples, and titre at 48 h was used as a proxy for fitness on each host type.

(f) Statistical analysis

Mixed linear models were used to assess the effects of fixed effect of selection treatment and random effects of population and block on viral fitness (package 'nlme', R 2014) [16]. When significant selection treatment effects were found, specific hypotheses were tested using pairwise contrasts of means and corrected for multiple comparisons using the sequential Bonferroni method (package 'multcomp', R 2015). Levene's test (package 'car', R 2015) was used to test for homogeneity of variance in fitness (log[viral titre]) across treatments. An outlier in treatment 4 was excluded when calculating variance for subsequent Pearson correlations. Kendall's rank correlation was used to test for a significant relationship between invasion rate of the novel host and evolved fitness. Non-parametric Kruskal–Wallis tests were used to test whether new alleles in evolved populations were distributed disproportionally across the five genes, and whether the number of mutations per genome varied across treatments. When significant effects were found, pairwise contrasts between genes were made using similar non-parametric methods correcting for multiple comparisons using the sequential Bonferroni method. Nucleotide diversities were calculated in R using the package PopGenome [17].

(g) RNA isolation, reverse transcription, polymerase chain reaction and genomic sequencing

Genomic viral RNA was isolated from 145 lineages (144 evolved populations plus the ancestor) using the QiaAmp Viral RNA mini kit (Qiagen). 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 eight primer pairs, generating overlapping PCR fragments 1–2.5 kb in length. Following purification with ExoI and Antarctic Phosphatase (NEB), the fragments were sequenced via dye termination (Sanger) at Yale University. Coverage was generated at two- to fourfold using overlapping fragments and multiple directional sequencing primers. This approach generates a consensus genomic sequence for each population, rather than a genome sequence derived from representative clones. It also allows for the detection of high-frequency minority variants at polymorphic sites, although variable loci at low frequency will be undetected by this method.

(h) Genome assembly and alignment

All sequences were reviewed following genome assembly using DNA Workbench 6 (CLC Bio). Where multiple quality sequencing reads resulted in strong secondary peaks at a single locus, the population was concluded to be polymorphic at the locus. Consensus sequences from the evolved populations were aligned to the ancestral sequence generated in this study.

3. Results

(a) Imaging reveals evenly mixed host environments

To examine the spatial composition of mixed host environments, CHO and BHK cells were fluorescently labelled, and mixed cultures were imaged across a range of relative cell proportions. The mixed cells formed a homogeneously mixed monolayer, with no evidence of higher order structure (i.e. cells preferentially binding to like cells) (electronic supplementary material, figure S1). These images demonstrated that the infecting virus populations experienced expected probabilities of host encounter in passages containing mixed host environments (e.g. in a 50% CHO monolayer, there should be roughly equal probability that progeny viruses exiting a cell encounter CHO versus BHK host cells).

(b) Treatment-specific patterns of fitness change

We assayed absolute fitness (48 h growth) for each lineage on CHO cells and on BHK cells after 25 passages of evolution. Nearly all virus populations evolved improved fitness on the novel host type (CHO) relative to the ancestral virus. Analyses showed a significant interaction between experimental treatment and evolved fitness on CHO cells, indicating that the different rates of CHO cell invasion held consequences for evolved fitness on this novel host ($F_5 = 9.2901$, $p < 0.0001$). There was a significant correlation between invasion rate of the novel host and evolved fitness on the novel host, with populations in treatments that experienced a more gradual invasion having higher fitness on average (Kendall $\tau = 0.19$, $p < 0.0001$) (figure 2*a*). For treatments where virus populations transitioned onto the novel host cell type, contrasts of means showed significant differences between the sudden-turnover treatment (treatment 1) and three of the treatments with gradual turnovers (treatments 2, 3 and 5; $p < 0.05$ in all cases). A significant difference between treatment 1 and treatment 4 was observed when the outlier was removed ($p < 0.01$). As expected, the control treatment in which virus populations were maintained on BHK cells for the duration of the experiment showed

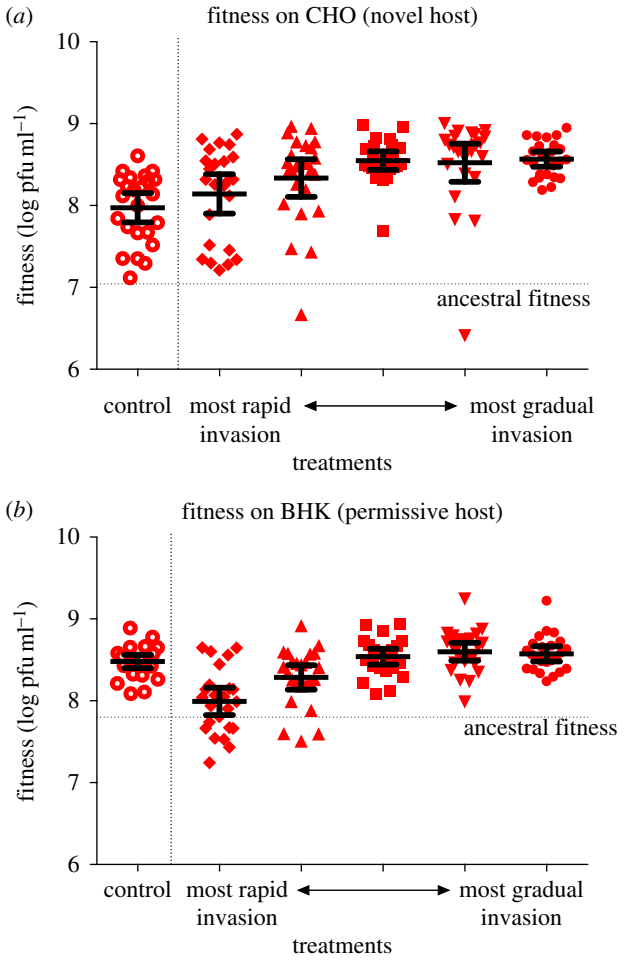

Figure 2. Evolved fitness on CHO and BHK by treatment. Each symbol represents the fitness of a single virus lineage on the cell type (mean of three trials): treatment 1 (diamonds), treatment 2 (up-triangles), treatment 3 (squares), treatment 4 (down-triangles), treatment 5 (closed circles) and treatment 6 (open circles). For each treatment, mean and 95% CI are shown. The dashed line represents the fitness of the ancestral strain (mean of nine trials). Control lineages (treatment 6) were evolved on BHK cells. (*a*) Fitness on CHO cells. (*b*) Fitness on BHK cells. (Online version in colour.)

lower mean fitness on CHO cells than all other treatments ($p < 0.01$ in all cases). The apparent treatment-specific differences in fitness on CHO cells were driven by significant differences in variance among populations within each treatment ($F_4 = 3.4568$, $p < 0.01$). Variance among replicate populations was negatively correlated with the number of transfers spent in mixed environments, meaning we observed lower variance in treatments with more gradual rates of environmental turnover (Pearson $r = -0.89$, $p = 0.04$). Therefore, populations more consistently reached high fitness on the novel host in response to more gradual invasion. Populations from the control BHK-only treatment also showed improved fitness on CHO cells, which suggests a correlated improvement for the two cell types.

There was also a significant interaction between experimental treatment and evolved fitness on BHK cells ($F_5 = 11.9807$, $p < 0.0001$). As expected, populations that encountered more BHK cells in the environment over the course of the experiment evolved higher fitness on BHK cells (Kendall $\tau = 0.35$, $p < 0.0001$) (figure 2*b*). Treatments with more gradual depletion of BHK cells evolved higher fitness on this cell type. In sudden-turnover treatment, in which populations were evolved solely on CHO cells, some populations fortuitously improved fitness on BHK cells via correlated response to selection, whereas others showed decreases relative to the ancestor. Significant differences in variance by treatment were observed ($F_4 = 8.1236$, $p < 0.001$), with variance among replicate populations correlated negatively with the number of transfers spent in mixed environments (Pearson $r = -0.89$, $p = 0.045$).

Two virus lineages showed reduced growth on the novel host relative to the ancestor. These lineages also presented low growth rates on BHK cells. While we attempted to control the bottleneck size across treatments and populations, we were unable to titre each lineage at every passage, and we based our imposed dilutions on sampling of a subset of lineages from each treatment. It is possible that these two populations experienced an unintentionally small bottleneck, in which case deleterious mutations may have gone to fixation.

(c) No observed trade-off between host types

To investigate whether there was a trade-off between growth on the two host types, the fitness of each evolved population on CHO cells was plotted against the fitness of each population on BHK cells (electronic supplementary material, figure S2). If there were a trade-off between growth on the two cell types, we would expect to see a negative correlation. In fact, fitness on CHO was positively correlated with fitness on BHK in all treatments where viruses transitioned onto CHO cells (Spearman treatment 1: $\rho = 0.5$, $p = 0.0096$; treatment 2: $\rho = 0.5$, $p = 0.0121$; treatment 3: $\rho = 0.6$, $p = 0.0017$; treatment 4: $\rho = 0.8$, $p < 0.0001$; treatment 5: $\rho = 0.7$, $p < 0.0001$). In the BHK-only treatment (treatment 6), there was no significant correlation between fitness on CHO and fitness on BHK (Spearman's $\rho = 0.2$, $p = 0.3134$). Thus, we observed no evidence for a trade-off between growth on CHO cells and BHK cells.

(d) Observed molecular changes and inferred phenotype–genotype associations

We identified a total of 362 new or polymorphic alleles at 338 unique loci in the 144 evolved populations, ranging from 1 to 12 loci per population (figure 3). Eleven of the recorded mutations were indels, seven of which occurred in non-protein coding regions of the genome. Of mutations in the coding regions at unique loci, 63.9% (207) were non-synonymous and 36.1% (117) were synonymous. Consensus changes were unequally distributed across the seven gene coding regions, particularly when accounting for relative gene sequence length ($\chi^2_8 = 545.7791$, $p < 2.2 \times 10^{-16}$). Consensus changes were most common in the E2 gene, with a new allele or polymorphic locus being identified on average every 13 bases. E2 is an envelope protein involved in cell recognition and attachment. In general, structural genes had higher rates of new alleles than non-structural genes, with affected loci identified on average every 34 bases in C, every 38 bases in E3, every 27 bases in 6K, and every 42 bases in E1, and every 58 bases in nsp1, every 42 bases in nsp2, every 37 bases in nsp3, and every 70 bases in nsp4. The number of mutations per genome did not vary significantly across treatments that transitioned onto CHO cells ($\chi^2_4 = 7.8612$, $p = 0.097$).

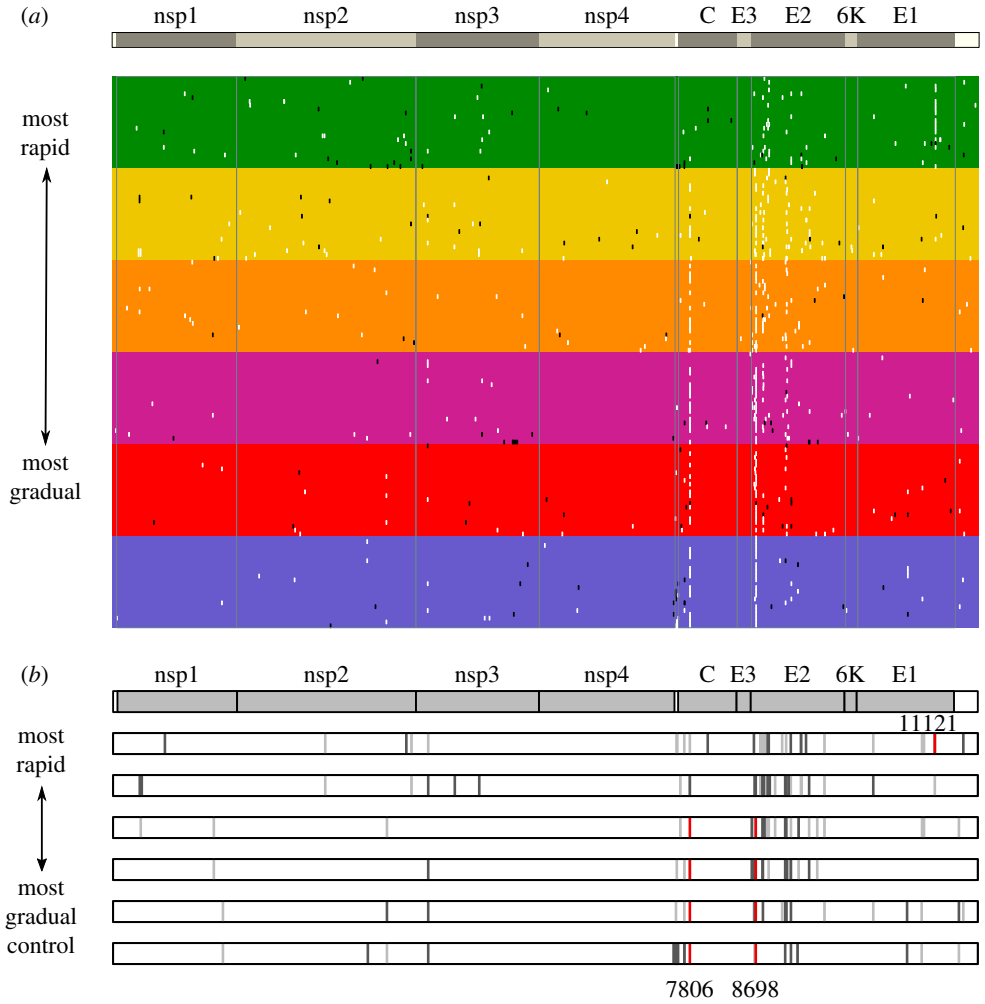

Figure 3. Molecular substitutions in evolved populations. (*a*) Location of molecular substitutions for all 144 evolved virus populations. The top bar represents the SINV genome, with coding regions for each gene shaded. Each colour block represents an experimental treatment, and each block is composed of stacked rows representing the 24 virus lineages in each treatment. Fixed mutations in each lineage are marked with white squares, and polymorphic sites are marked with black squares. Treatment 1 (green), treatment 2 (yellow), treatment 3 (orange), treatment 4 (pink), treatment 5 (red) and treatment 6 (blue). (*b*) Summary of convergent substitutions by experimental treatment. The top bar represents the SINV genome, with coding regions for each gene shaded. Each experimental treatment is represented by one bar. Molecular substitutions observed in more than one population in the total dataset are marked in the treatments in which they were observed. Substitutions observed in just one population within a treatment but at least once more in another treatment are marked in light grey. Substitutions observed in two to nine replicate populations within a treatment are marked in dark grey. Substitutions observed in 10 or more replicate populations within a treatment are marked in red. The three most commonly observed mutations in the dataset are labelled by their location in the genome (7806, 8698, 11121).

The most widely distributed new alleles segregated by treatment. In the treatment with the most rapid invasion (evolved on 100% CHO cells for the duration of the experiment), the most common mutation was T11121A in the E1 envelope protein (figure 3*b*). Twelve of 24 populations in this treatment converged on this mutation. However, this mutation appeared only once outside of this treatment, in the treatment with the second most rapid invasion. The other most widely mutated loci occurred primarily in treatments that had gone through a more gradual invasion and the BHK-only control treatment. These mutations were I7806V and a mutation at E8698G (figure 3*b*). The substitution at 7806 leads to an amino acid substitution in the capsid protein, and occurred in 56 out of 144 populations. The I7806V mutation appeared only once in the most rapid invasion treatment, seven times in treatment 2, 12 times in treatment 3, 13 times in treatment 4, 10 times in treatment 5, and 13 times in the BHK-only control treatment. Seventy-four of 144 evolved populations had a consensus substitution or polymorphism at 8698. In the majority of these cases (65 of 74), an A to G mutation led to an E to G amino acid substitution. An additional seven lineages had an E to A amino acid substitution at this site, and two populations showed an E to V substitution. Mutations at 8698 occurred only in populations that had gone through a gradual invasion, and in the BHK-only control treatment. These loci were affected in nine lineages in treatment 2, 13 lineages in treatment 3, 17 lineages in treatment 4, 15 lineages in treatment 5, and 20 lineages in the BHK-only control treatment.

(e) Nucleotide diversity

Corrected nucleotide diversity is the average number of pairwise base differences between two genomes in a sample (in this case, a treatment), divided by the number of bases in the genome. We used this statistic to estimate the genomic divergence among consensus sequences within each treatment. For treatments that transitioned onto CHO cells, we found that nucleotide diversity among populations within a treatment was correlated with the rate of novel host invasion

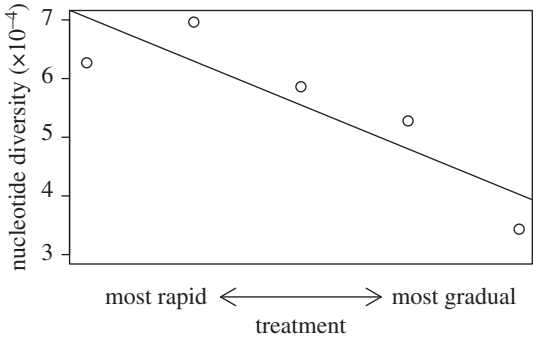

Figure 4. Nucleotide diversity for each treatment. For each treatment that experienced an invasion of the novel host, the corrected nucleotide diversity among consensus sequences of replicate lineages is plotted.

(Spearman's $\rho = -0.9$, $p = 0.0374$). Nucleotide diversity was highest in response to the sudden host invasion, and decreased as the invasions occurred more gradually (figure 4).

4. Discussion

We found that when RNA virus populations were faced with the invasion of a novel host, the rate of host invasion influenced the predictability of evolutionary outcomes at both the phenotypic and genomic levels. More gradual rates of host invasion more consistently resulted in high viral fitness achieved on the novel host. Gradual host invasions were also correlated with lower genetic divergence among replicate virus populations within a treatment. In other words, treatments that resulted in less variable phenotypes also produced less variable genotypes. We found that genetic parallelism was common, but that convergent alleles segregated by treatment. Epistatic interactions may contribute to constrained outcomes for viruses evolving in mixed host environments.

Previous experimental work on adaptation in directionally changing environments has focused on continuous abiotic environmental variables, such as phosphate or antibiotic concentration. These experiments have demonstrated that more gradual environmental change tends to result in populations that are better adapted to their final environment or less likely to go extinct [8,9,12]. *Chlamydomonas* populations evolved in environments with increasing phosphate concentrations reached fitter endpoints when the environment changed more gradually [12], and bacterial populations evolved in response to increasing antibiotic concentrations more consistently avoided extinction and reached high endpoint fitness when adapting to gradual change [9]. Our results reaffirm the finding that more gradual rates of change tend to result in better-adapted populations, and we demonstrate that this effect holds true for populations responding to change in a patchy biotic environmental variable.

To date, it has been difficult to draw strong conclusions about how the rate of environmental change affects the repeatability of phenotypic and genotypic evolution. Theory predicts that gradual environmental change will result in more diverse evolutionary outcomes, as adaptive walks in these environments are less constrained in their first mutational steps [4]. Empirical work with algae supports this prediction [12]. In the first study to explore the genetic underpinnings of adaptation to gradually changing environments, higher nucleotide diversity was observed for a bacterial antibiotic resistance gene in treatments with more gradual environmental change [9]. However, another study of bacterial adaptation to varying rates of antibiotic increase showed that a sudden environmental change resulted in more diverse resistance phenotypes [8]. Our results show greater diversity in both phenotype and genotype in response to a sudden environmental change. Populations evolving in gradually changing environments may be constrained by having to persist in intermediate environments, leading to less divergence [6]. This may be particularly true in patchy host environments, where multiple selection pressures may be acting in mixed environments. Our study is, to our knowledge, the first to sequence whole genomes of populations evolved in response to different rates of environmental change, providing a more complete picture of how the rate of change influences genetic divergence at the genomic level.

In experimental evolution, the occurrence of parallel substitutions across replicate lineages is generally taken as strong evidence that the convergent alleles are adaptive. In the treatment with the sudden shift onto the novel host, the T11121A substitution occurred in 12 out of 24 populations. We take this as strong evidence that this allele in the E3 gene is adaptive on CHO cells. We note that while this substitution was inferred to be adaptive, its presence or absence alone did not explain the variation in fitness on CHO cells among lineages within this treatment.

Because the T11121A substitution was the most commonly converged upon substitution among lineages adapting to a sudden shift onto CHO cells, we were surprised to find that this substitution almost never occurred in populations that shifted gradually onto the novel CHO host cells. Out of 96 populations that experienced some gradual rate of CHO cell invasion, only one population had the T11121A substitution in its consensus sequence. This population was in the treatment with the second most rapid invasion. The T11121A substitution never occurred on control populations evolving solely on BHK cells, which suggests that this allele is specifically beneficial to growth on CHO cells. Our observations suggest that historical contingency is an important determinant of evolved genotype, and that epistasis constrains what genotypes are available to evolving populations. Even in the treatment where populations evolved for just five passages in mixed cell environments, and then for 20 passages solely on CHO cells, only one of 24 populations found the T11121A substitution. We infer that the effect of the T11121A substitution is highly dependent on the genetic background.

The other two most widely distributed alleles, I7806V and E8698G, were common in the BHK-only control treatment as well as treatments that had experienced mixed cell environments. Because of the prevalence of these mutations in the BHK-only control and their near total absence in the CHO-only treatment, we infer that these mutations were beneficial to growth on BHK cells. Moreover, we hypothesize that the I7806V substitution in the capsid and the E8698G substitution in E2 interact epistatically, with the capsid mutation being beneficial only in a background with the E2 substitution. Fifty-five out of the 56 times the I7806V substitution was observed, it was in a genetic background that contained the E8698G allele. The one lineage containing I7806V without E8698G occurred in the treatment with the most rapid invasion (i.e. only selected on CHO cells). It appears that in environments with BHK cells, an E8698G substitution may be necessary to pave the way for the beneficial I7806V substitution.

These results add to the growing body of evidence showing the prevalence and importance of epistatic interactions in evolving populations. Epistatic effects may close off certain adaptive pathways depending on genetic background, as with the T11121A substitution, or gaining one mutation may facilitate access to an additional beneficial substitution, as with the E8698G and I7806V substitutions. RNA viruses may experience particularly rugged fitness landscapes owing to constraint both in protein coding and functional RNA secondary structure. This added level of epistatic constraint, combined with their small and often easily manipulated genomes, makes RNA viruses a valuable model for the study of epistatic interactions and adaptation.

Our results show that the ability of a virus to adapt to a novel host depends upon the rate of environmental turnover, which can also be framed as the depletion of a preferred host. At the cellular level, this is relevant for tumour cells being treated with oncolytic viruses or HIV viruses expanding their host range as their preferred host cell type dwindles in prevalence. Our results suggest, for example, that if a tumour is treated with an oncolytic virus, the rate at which tumour cells are killed may influence the likelihood of virus emergence on unintended host cells.

In conclusion, we provide evidence that the rate of novel host invasion effects adaptive outcomes for an RNA virus at both the phenotypic and genomic level. Adaptive walks in our study are more repeatable when the novel host invaded the environment more gradually. This study adds further incentive for experimental evolution to expand its horizons beyond the standard ecological scenario of a sudden environmental change [3]. Viruses are often evolving in complex host communities that are variable in space and time, and a focus on simple environments may miss important aspects of adaptation to hosts in the natural world [18]. Finally, our results add to a growing number of studies highlighting the importance of epistasis and historical contingency in shaping adaptive walks, and suggest that RNA viruses are a powerful model for studying epistasis going forward.

Data accessibility. Data are available on Dryad (http://dx.doi.org/10.5061/dryad.9b30b) and genomic data are available in GenBank (KT121582–KT121726).

Authors' contributions. V.J.M. conceived of the study, carried out the molecular and tissue-culture laboratory work, conducted the data analysis, carried out the sequence alignments and drafted the manuscript. S.Y.M. contributed to sample preparation for genome sequencing. P.E.T. contributed to design and interpretation of experiments and drafting the manuscript. All authors gave final approval for publication.

Competing interests. We have no competing interests to declare.

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