

Arbovirus evolution *in vivo* is constrained by host alternation

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The intrinsic plasticity of RNA viruses can facilitate host range changes that lead to epidemics. However, evolutionary processes promoting cross-species transfers are poorly defined, especially for arthropod-borne viruses (arboviruses). In theory, cross species transfers by arboviruses may be constrained by their alternating infection of disparate hosts, where optimal replication in one host involves a fitness tradeoff for the other. Accordingly, freeing arboviruses from alternate replication via specialization in a single host should accelerate adaptation. This hypothesis has been tested by using cell culture model systems with inconclusive results. Therefore, we tested it using an *in vivo* system with Venezuelan equine encephalitis virus (VEEV), an emerging alphavirus of the Americas. VEEV serially passaged in mosquitoes exhibited increased mosquito infectivity and vertebrate-specialized strains produced higher viremias. Conversely, alternately passaged VEEV experienced no detectable fitness gains in either host. These results suggest that arbovirus adaptation and evolution is limited by obligate host alternation and predict that arboviral emergence via host range changes may be less frequent than that of single host animal RNA viruses.

adaptation | RNA virus emergence | venezuelan equine encephalitis virus

The emergence of pathogenic RNA viruses is often associated with their genomic plasticity and alterations in the environment that lead to novel host contacts. Nearly 50 new human pathogens, mostly RNA viruses, have been identified in the last quarter century, many as a result of introductions into human populations (1). Cross-species transfers often mediate pathogen emergence via the stochastic generation of virus variants able to replicate in a new host in the appropriate ecological setting. Several RNA viruses, including HIV (2), SARS coronavirus (3, 4), and the arbovirus dengue virus (DENV) (5) have caused recent epidemics by changing their host ranges to increase infections of humans.

Evolutionary processes that mediate changes in host range are poorly understood. For most RNA viruses, it is unclear whether the expansion of host range involves adaptation to novel host(s) or preexisting infectivity. For example, phylogenetic analyses indicate that DENV emerged via a transfer from nonhuman primate to human hosts (5, 6). Given the similar selection pressures and evolutionary rates shared by other RNA arboviruses (7), evidence from DENV studies suggests that emergence of other arboviral pathogens via adaptation for urban transmission is also possible. Adaptation to the urban vector *Aedes albopictus* may have expanded a 2005–2006 outbreak of Chikungunya virus in Reunion Island (8, 9) that subsequently circulated among humans in the absence of other amplifying hosts. Other tropical arboviruses that produce human viremia, including Venezuelan equine encephalitis virus, may also have the potential for similar urbanization (10).

Arboviruses are principally transmitted horizontally between arthropod vectors and vertebrate reservoir hosts. The majority of arboviruses are RNA viruses that lack polymerases with proof-reading activity and thus exhibit error frequencies of $\approx 10^{-4}$ (11). Their high mutation frequencies, rapid replication, and large population sizes allow these viruses to rapidly adapt to fluctuating environments. However, sequence comparisons of RNA arbovi-

rus reveal that they are relatively stable in nature, and genetic studies suggest that strong purifying selection dominates their evolution (6, 12). This stability may result from the requirement for replication in two disparate hosts, which presents conflicting demands for replication and adaptation and which could constrain adaptation to either host alone by imposing a fitness cost where adaptations are antagonistic (13). According to this hypothesis, freeing RNA arboviruses from alternate host replication should facilitate rapid adaptation to individual hosts.

Experimental microbial evolution provides an opportunity to study mechanisms of fitness trade-offs and to understand the unique ability of RNA arboviruses to simultaneously evolve in alternate hosts. The alphavirus Ross River virus (*Togaviridae: Alphavirus*) exhibits increased neurovirulence in mice after serial brain passages, but shows phenotypic stability after alternating mouse and mosquito infections (14, 15). *In vitro* model fitness studies measuring relative reproductive success of arboviruses alternately or serially passaged in vertebrate and invertebrate cells (16–19) show three general trends: (i) fitness gains in the cell used for passage [except as observed by Cooper and Scott (16)] and losses in the bypassed cell [Sindbis virus (SINV), Eastern equine encephalitis virus (EEEV), and Vesicular stomatitis virus (VSV)]; (ii) impaired replication in novel cells (VSV); and (iii) successful adaptation to fluctuating cell environments [EEEV (19), SINV (17), VSV (18)]. Taken together, these *in vitro* results support the hypothesis that fitness constraints differ in vertebrate and insect cells and may be virus-specific but do not indicate that arbovirus fitness is constrained by alternating host transmission cycles. However, artifactual factors may compromise *in vitro* model systems of arbovirus adaptation. For example, serial passaging of the alphaviruses SINV (20) and VEEV (21) in baby hamster kidney (BHK) cells results in adaptive attenuating mutations associated with adaptation to use heparan sulfate as a receptor via the acquisition of charged amino acid residues in the E2 envelope glycoprotein. Thus, serially *in vitro* passaged viruses undergo artificial adaptation to associate with host cell molecules that are not selective factors *in vivo*.

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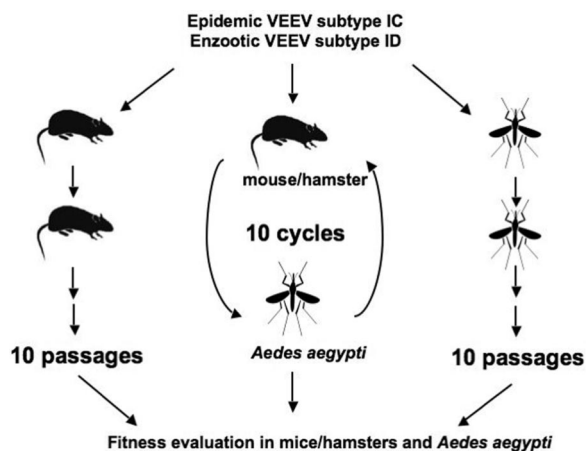


Fig. 1. Experimental design for VEEV *in vivo* adaptation studies. VEEV subtype IC or ID strains were serially passaged 10 times in vertebrates (Left) or *A. aegypti* mosquitoes (Right) to artificially bypass one host, or alternately passaged (Center) to simulate natural transmission. The fitness of VEEV derived from these passage series compared to parent VEEV was determined by direct replication comparison or competition assay.

We sought to test our central hypothesis that the alternating host transmission cycle of arboviruses constrains their ability to adapt to new hosts and vectors. To circumvent limitations of cell culture models, we passaged VEEV (*Togaviridae: Alphavirus*) either alternately or serially in *Aedes aegypti* mosquitoes or laboratory rodents and then compared the fitness of progeny viruses to that of the parental isolate. VEEV, with its single stranded positive-sense nonsegmented ≈ 11.4 -kb RNA genome that encodes seven major proteins, causes outbreaks of equine and human disease in Central and South America (10). On at least four independent occasions, the emergence of VEEV from enzootic progenitor viruses was mediated by changes in host range via adaptation for efficient amplification in equids (22–25) and/or increased infectivity for mosquito vectors (26, 27). During epidemics, viremic horses can infect large populations of mammalophilic mosquito vectors that subsequently feed on people in agricultural habitats. Because VEEV produces viremia in humans comparable to that in equids (28), adaptation for increased transmission by an urban vector such as *A. aegypti* could result in a DENV-like epidemiology and VEEV epidemics could become widespread in Latin America, with devastating public health consequences. Thus, VEEV serves both as an excellent theoretical model to study constraints on the evolution of arbovirus host range changes and a practical model to assess the potential for urbanization in the neotropics.

Results

To assess the influence of host alternation on arbovirus adaptation to new hosts or vectors, two strains of VEEV were passaged serially in mice or hamsters alone, in *A. aegypti* mosquitoes alone, or in an alternating transmission cycle (Fig. 1). Enzoitic subtype ID strain 8131 was used because it circulates in Iquitos, Peru, where urban VEEV is regularly detected (29). Strain 3908, a 1995 subtype IC isolate from Venezuela, was used because it was isolated from a human during the last major epidemic in a location amenable to urban circulation near Maracaibo (30). Because rodents are reservoir hosts of VEEV, mice and hamsters were selected as convenient laboratory hosts. *A. aegypti* was chosen to assess the potential of VEEV to urbanize because (i) it is not a natural vector like *Culex (Melanoconion)* spp. that has already coevolved with VEEV, providing the potential for rapid adaptation; (ii) it is susceptible to infection; and (iii) VEEV epidemics occur in Latin American cities where this mosquito is abundant. Natural *Culex (Melanoconion)* spp. vectors were not used because they feed poorly on artificial

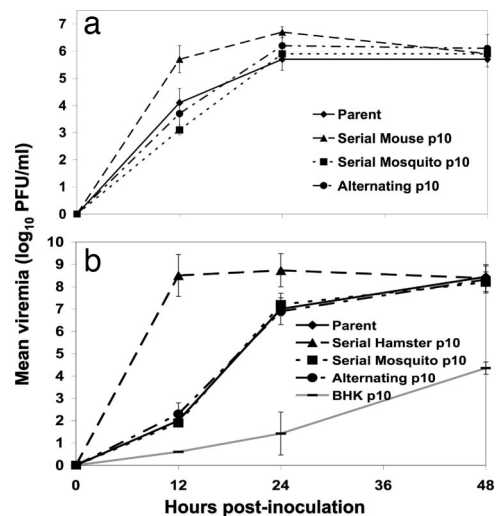


Fig. 2. Mean viremias in National Institutes of Health Swiss mice (VEEV ID strain 8131) (a) or Syrian golden hamsters (VEEV IC strain 3908) (b) inoculated with doses ranging from 3.2 to 3.5 \log_{10} PFU/ml (ID) or 2.9–3.1 \log_{10} PFU/ml (IC) of parent, alternating p10, mouse/hamster p10, mosquito p10, or BHK p10. Mice ($n = 6$) and hamsters ($n = 3$) were bled at each time point; mean viremias are presented. Error bars indicate standard deviations.

blood meals. Fitness for mosquito infection and replication were tested by assays of two different stages subject to potential selection; overall infection rates (because initial infection is the main restriction on VEEV transmission efficiency); and body titers after extrinsic incubation. Ideally, mosquito saliva would have been collected from mosquitoes and introduced into artificial blood meals to optimally control serial mosquito passages. However, VEEV saliva titers rarely exceed 1,000 PFU (31). These titers, even from mosquito pools, would be insufficient to maintain serial mosquito passages, so pooled whole mosquito bodies were used to generate sufficient blood meal titers. To compare *in vivo* results with *in vitro* model systems used previously, VEEV strain 3908 was also passaged serially in baby hamster kidney (BHK) cells or alternately between BHK and RML12 *A. aegypti* cells. Serial passage in RML12 cells alone could not be sustained because replication titers were not adequate to maintain multiplicities of infection (MOI) of 0.1.

To assess fitness changes after serial passages, replication kinetics in vertebrate hosts (Fig. 2) and infection rates (Table 1) and virus titers in mosquitoes were compared. Glycosylation differences in VEEV produced by mosquito versus vertebrate cells can affect pathogenesis in rodents (32). To control for these differences and differences in the composition of blood meals offered to mosquitoes (e.g., triturated mosquito pools had little or no dilution and the serum of infected rodents probably contained host factors that are not present in normal serum) the first passage in each series rather than the parent strain was compared to the 10th passage.

Fitness in Vertebrates. Alternating passage. Enzoitic VEEV strain 8131 alternately passaged 10 times (p10) in mice and *A. aegypti* mosquitoes generated mean viremias that were statistically similar to the parent virus (p1) (Fig. 2a; $P > 0.05$, ANOVA) when assayed 12, 24, or 48 h postinfection (PI). Mice infected with p1 of strain 8131 survived an average of 8.0 ± 1.0 days PI and animals inoculated with alternating p10 survived 7.8 ± 0.4 days PI (data not shown). Epidemic VEEV strain 3908 alternately passaged 10 times in hamsters and mosquitoes generated the same viremia profile as the parent (Fig. 2b). Hamsters infected with parental strain 3908 and alternating p10 both survived an average of 2.8 ± 0.5 d PI (data not shown). These results indicated that 10 alternating cycles did not influence VEEV viremia or average survival time in rodent hosts.

Table 2. Competition fitness assays for VEEV strain 3908 in subcutaneously infected hamsters

Virus competition	Final/original ratio of passaged:surrogate parent virus \pm SD
3908 Parent vs. <i>Bsp E1</i> mutant	0.93 \pm 0.1
Alternating p10 vs. <i>BspE1</i> mutant	1.12 \pm 0.3
Hamster p10 vs. <i>BspE1</i> mutant	5.32 \pm 1.1*
Mosquito p10 vs. <i>BspE1</i> mutant	1.01 \pm 0.0
BHKp10 vs. <i>Bsp E1</i> mutant	<0.02*

Fitness differences between VEEV subtype IC strain 3908 marked surrogate parent (*BspE1*-mut) and passaged VEEV IC. Each competition assay was conducted in at least three hamsters. A ratio of 1 indicates that the two competing viruses have equal fitness. A value <1 indicates that the passaged virus is less fit than the surrogate parent. A ratio of >1 means that the passaged virus is more fit than the parent.

*Ratios that significantly deviate ($P < 0.05$) from a neutral fitness of 1 (Student's *t* test).

ablation of a *BspE1* restriction endonuclease site, using a synonymous mutation. The location of this site (genomic nucleotides 8345–8350) was in a region of the capsid protein gene not known to include regulatory elements under direct RNA sequence selection (34).

First, we verified that the marked surrogate parent (*BspE1*mut) exhibited neutral fitness compared to the wild-type parental virus in hamsters. Mixtures of the wild-type and *BspE1*mut were maintained at stable ratios after vertebrate passages *in vivo* or BHK and mosquito cell culture passages (data not shown), indicating that the genetic marker did not affect fitness. However, pilot studies in mosquitoes allowed to engorge on mixtures of the marked and wild-type viruses indicated that, because a very small number of VEEV virions initiate mosquito midgut infection after an infectious blood meal (35), the outcome of competition is sometimes stochastic (i.e., small population sizes can reduce the efficiency of natural selection). Therefore, competition assays were restricted to the rodent hosts.

Competition assays in hamsters (Table 2, $n = 3$ hamsters per competition) indicated that the ratio of alternating p10 VEEV IC strain 3908 to the 3908*BspE1*mut did not change significantly after coinfection of hamsters, indicating 10 alternating passages *in vivo* did not alter fitness in the vertebrate host. Similarly, 10 serial mosquito passages did not affect fitness in hamsters. In contrast, hamster p10 was five times more fit than the 3908*BspE1*mut in hamsters. As expected, the control BHK p10 strain showed a significant decrease in fitness for replication in hamsters.

Competition fitness assays also indicated significant increases in replication in BHK cells after BHK cell passaging and alternating BHK-RML12 cell passages (Table 3). In RML12 cells, BHK-adapted strain 3908 showed a significant reduction in fitness, whereas the alternating BHK-RML-passaged VEEV exhibited neutral fitness.

Sequence Analyses. Consensus sequences of *in vivo* passaged VEEV were determined to generate preliminary data on potential determinants of adaptation and were compared with parental sequences to determine mutations that may have mediated the observed changes in phenotypes. VEEV strain 8131 accrued four consensus synonymous mutations after 10 alternating passages and VEEV strain 3908 developed three such mutations [supporting information (SI) Table S1]. Despite producing a higher mean viremia at 12 h PI (Fig. 2a), the serial mouse p10 consensus 8131 sequence (parent GenBank accession no. DQ390224) accrued only one mutation. This nucleotide change occurred at genome position 6174 in the nsP4 polymerase gene and encoded an arginine (parent) to serine (mouse p10) change. To determine when the mutation arose in the mouse passage series, viral RNA was extracted from serum of every second mouse passage, and RT-PCR amplicons flanking nucleotide

Table 3. Competition fitness assays for VEEV strain 3908 in cell cultures

Virus competition	Cell in which competition occurred	Final/original ratio of passaged:surrogate parent virus \pm SD
3908 parent vs. <i>BspE1</i> mutant	BHK	0.98 \pm 0.05
BHK p10 vs. <i>BspE1</i> mutant		8.48 \pm 1.39*
<i>In vitro</i> alternating p10 vs. <i>BspE1</i> mutant		4.73 \pm 0.59*
3908 parent vs. <i>BspE1</i> mutant	RML12	0.93 \pm 0.04
BHK p10 vs. <i>BspE1</i> mutant		0.08 \pm 0.02*
<i>In vitro</i> alternating p10 vs. <i>BspE1</i> mutant		1.15 \pm 0.24

Fitness differences between VEEV subtype IC strain 3908 marked surrogate parent (*BspE1*-mutant) and cell culture passaged VEEV IC. Each competition assay was conducted in three cell culture replicates. A ratio value of 1 indicates that the two competing viruses have equal fitness. A value <1 indicates that the passaged virus is less fit than the surrogate parent. A ratio of >1 means that the passaged virus is more fit than the parent.

*Ratios that significantly deviate ($P < 0.05$) from a neutral fitness of 1 (Student's *t* test).

6174 were generated and sequenced. Sequencing chromatograms (Fig. S1a) revealed that a nucleotide mixture including the serine codon (G) was present as early as mouse p4, and the serine codon increased in frequency with additional passages, indicating positive selection. This change alone may have been sufficient to induce higher viremia 12 h PI compared to that generated by VEEV 8131 passaged once in mice. Hamster p10 of strain 3908 also contained one consensus nsP4 amino acid substitution (Met \rightarrow Leu) compared to its parent (GenBank accession no. U55350). Unexpectedly, despite observed phenotypic differences, 10 serial mosquito passages did not result in any consensus nucleotide changes in the genome of either VEEV strain compared to its parent. A mixed nucleotide population was observed at synonymous genomic nucleotide 123 in the nsP1 gene of strain 8131 mosquito p10. Sequencing chromatograms (Fig. S1b) revealed that the mutant A nucleotide was present as a minority population beginning with mosquito passage 8, and that the frequency of the A nucleotide (reflected in the height of the minority chromatogram peak) increased between mosquito passage 8 and 10, also indicative of positive selection. Further mosquito passages might be interesting to confirm these findings.

Serial passage of strain 3908 in BHK cells resulted in a Gly \rightarrow Lys substitution at amino acid position 3 in the E2 glycoprotein within the furin cleavage site implicated in differential binding to heparan sulfate (21). The *in vitro* alternating passages resulted in a Thr \rightarrow Lys substitution at E2 position 119, again suggestive of adaptation for glycosaminoglycan binding.

Discussion

Because host range changes can mediate the emergence of RNA arboviral diseases, we determined the effect of the alternating host transmission cycle on arbovirus adaptation. In support of the hypothesis that host alternation constrains the ability of arboviruses to adapt, freeing VEEV from replication in either the vertebrate or mosquito host (thereby allowing it to specialize in a single host) facilitated adaptation during *in vivo* passages. In contrast, *in vitro* alphavirus passages permit simultaneous adaptation to both vertebrate and mosquito cell lines (17, 19), underscoring the limitations of cell culture model systems to reproduce natural, *in vivo* selective systems. Serial *in vivo* mosquito passages resulted in enhanced mosquito infectivity, but equal or reduced viremia in vertebrates, indicating that adaptation for mosquito infection was host-specific. Bypassing the arthropod via serial vertebrate passages increased early viremia and reduced mosquito infectivity, consistent with the hypothesis that specialization enhances adaptation in a host-specific

manner. Adaptation to the vertebrate host appeared to be more rapid or efficient than adaptation to mosquitoes, possibly because more VEEV replication occurs in the former; the population size of VEEV in the rodent host approaches $\approx 9 \log_{10}$ PFU/ml serum but, in a mosquito, rarely exceeds $7 \log_{10}$ PFU/ml (10).

Our results support the theory that arboviruses, which replicate obligately in both invertebrates and vertebrates, are evolutionarily constrained and that vector-borne transmission imposes fitness trade-offs for replication in both hosts. Alternating *in vivo* passaging did not significantly increase mosquito infectivity or elevate rodent viremia. Although adaptation was not detected, infection and replication of alternately passaged VEEV was not impaired in either host. Serial passage of VEEV in BHK hamster cells resulted in significant fitness declines *in vivo* for both mosquito and vertebrate hosts, whereas alternating *in vitro* passages stabilized fitness in both hosts. The loss of virulence after BHK cell passages underscores the problems with these *in vitro* model systems.

A possible explanation for the difference between *in vivo* and *in vitro* results is that VEEV passaged *in vivo* replicates in more than one cell type before peak viremia in rodents, or during 10 days of extrinsic incubation in mosquitoes, before transmission transfer to the alternate host. However, arboviruses passaged *in vitro* only replicate in a single cell type and heparan sulfate is common in vertebrate and invertebrate cell culture systems. Strong purifying selection imposed by replication *in vivo* within multiple cell types may be commonplace among RNA viruses.

We conducted preliminary sequence analyses to determine changes in the VEEV consensus sequences after passages. Sequence data revealed that the genetic changes that mediated VEEV adaptation to new hosts were slight; a single nsP4 amino acid change in serial vertebrate p10 for both VEEV strains was probably sufficient to elevate early viremia, and a nucleotide mixture at genome position 123 (nsP1) in VEEV strain 8131 mosquito p10 probably enhanced mosquito infectivity. The gradual replacement of one nucleotide by another through the passage series (Fig. S1) suggested the influence of positive selection rather than genetic drift. Because the nsP4 gene has not been implicated in adaptation to new hosts, its role in determining the rodent viremia phenotype and mosquito infectivity should be further explored through reverse genetic strategies. Although the alphavirus nsP1 gene encodes a membrane-associated protein that controls minus-strand RNA synthesis and acts as a methyltransferase and guanyltransferase (34), none of these functions are encoded in the genome region (nucleotide 123), where we observed positive selection. Because this genome region is in the vicinity of conserved, secondary structure elements that are important for alphavirus replication (34), the RNA sequence itself may have been selected in our experiment.

The small number of nucleotide changes detected in the consensus sequences of our *in vivo* adapted viruses is inconsistent with higher numbers of mutations observed for *in vitro* adapted alphaviruses (17, 19) and suggests that *in vivo* environments are less permissive for rapid sequence change. The consensus sequences we determined only reflect the majority nucleotide at a given position and do not represent the distribution of sequence variants within the viral population. It is possible that other mutations in our adapted viruses, present at low frequencies and undetected by using sequencing of RT-PCR amplicons [like those observed for other RNA arboviruses (37)], are involved in adaptation or that the mutant spectra expand after serial passages. Because minority sequence variants can exhibit dominant phenotypes in a viral populations (38), further sequencing of biological or molecular clones derived from our adapted VEEV populations are needed.

In summary, our results support the hypothesis that alternating transmission between vertebrates and invertebrates constrains host range changes by arboviruses. Despite the potential for RNA viruses to rapidly adapt via large population sizes, high mutation rates, and short replication times, arboviruses may be more con-

strained than single-host RNA viruses in their adaptability. The acquisition of mutations beneficial for vertebrates may impose fitness costs for replication in invertebrates and vice versa, a process that could restrict genetic change and enforce purifying selection. Even if adaptation to new hosts is limited by the arbovirus transmission cycle, critical adaptation events involving single point mutations shown to mediate VEEV adaptation to either mosquito vectors (26) or equine amplification hosts (22) do occur in nature, leading to devastating VEE epidemics. Ecological and epidemiological limitations probably play an important role in shaping these emergences, and deserve equal attention.

Materials and Methods

Viruses. To represent both epidemic and enzootic varieties, two strains of VEEV were used for adaptation experiments: (i) strain 3908, a subtype IC epidemic-type VEEV isolated from a febrile human during a 1995 epidemic in Venezuela that was passaged once in C6/36 mosquito cells before infectious cDNA clone construction, and (ii) strain 8131, an endemic-type ID strain isolated from a febrile human in Peru in 1998 that was passaged once in BHK cells. For VEEV strain 8131 that was not derived from an infectious clone, we used plaque-purified virus to generate a stock that initiated each transmission series.

Virus Assays. Serum, blood meal, and mosquito samples were tested for VEEV by standard plaque assay on Vero cells (39). Limits of detection ranged from 0.6 to $1.9 \log_{10}$ PFU/ml.

Vertebrates and Mosquitoes. For mosquito studies, 5- to 10-day-old first generation *A. aegypti* reared from wild mosquitoes caught in Galveston, Texas, were used. The range of ages across cohorts was comparable because mosquitoes were collected from the same cage and randomly assigned to groups. Mosquitoes were maintained in a BSL-3 insectary in humidified boxes inside 28°C incubators with a 12 h:12 h light:dark cycle. Adult female Syrian golden hamsters were used for subtype IC transmission cycles and 6- to 8-week-old adult female National Institutes of Health Swiss mice (Harlan) were used for subtype ID transmission cycles. Mice and hamsters were used as model reservoirs because they develop viremia levels comparable to those in subtype IC VEEV-infected equids or subtype ID-infected rodent hosts (10). For mosquito feeds, rodents were anesthetized with 40–85 mg/kg sodium pentobarbital. Manipulations of vertebrates were carried out by using a protocol approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee in accordance with recommended guidelines.

Transmission Cycles. Alternating cycles. To simulate natural transmission, VEEVs were alternately passaged in vertebrates and *A. aegypti* mosquitoes (Fig. 1 Center). To initiate each cycle, a hamster or mouse was inoculated s.c. in the right thigh with $\approx 1,000$ PFU of VEEV strain 3908 or 8131, respectively. Twenty-four hours PI, the time of peak VEEV viremia, cohorts of 50–100 *A. aegypti* were presented to anesthetized vertebrates and allowed to feed for 1–2 h. Engorged mosquitoes were held in pint containers with 10% sucrose ad libitum for 9–13 days, sugar starved for 24 h, and then allowed to refeed on a naive animal. Successful VEEV transmission to the naive animal initiated the subsequent cycle and was verified by recovery of VEEV from the serum 24 h after refeed or by fatal disease in the animal 3–6 days later. A group of uninfected mosquitoes was allowed to feed at the peak viremia of the second animal to continue the natural alternating cycle. Mosquitoes were killed after the refeed by freezing, and bodies and legs were individually homogenized (Retsch MM300 homogenizer) in minimum essential medium (MEM) with 20% FBS and frozen at -80°C until plaque assay. Infection was determined by recovery of virus from the homogenized body and dissemination from the alimentary track into the hemocoel was determined by recovery of virus from the legs. The infection rate was recorded as the fraction of virus-positive bodies divided by the total number of bodies and the disseminated infection rate is the number of virus-positive legs divided by the total number of fed mosquitoes. Virus-positive bodies from the 10th feed were pooled and used for sequence and fitness assays.

Serial vertebrate cycles. To initiate serial vertebrate infections (bypassing the arthropod vector; Fig. 1 Left), VEEV subtype IC and ID strains from the same stocks used for alternating transmission cycles were inoculated as above into mice or hamsters. Infected animals were housed individually until peak viremia and then anesthetized and bled via the retro-orbital sinus. Serum was diluted 1:100 in MEM with 5% FBS for administration to a naive animal.

Serial mosquito cycles. VEEV IC and ID strains were passaged 10 times in mosquitoes (Fig. 1 Right) to determine whether serial passaging in arthropods results in adaptation to mosquitoes and decreased fitness in vertebrates. Virus

stocks diluted to $\approx 6 \log_{10}$ PFU/ml were placed in a 1:1 mixture with artificial blood meals consisting of 1% (weight/vol) sucrose, 20% (vol/vol) FBS, 5 mmol of ATP, 33% (vol/vol) PBS-washed sheep blood cells (Colorado Serum), and 33% (vol/vol) MEM. Infectious blood meals were warmed in a membrane feeder (Discovery Workshops) and loaded into artificial membrane feeders on cartons containing *A. aegypti*. After 10 days incubation of engorged mosquitoes, the bodies of survivors were pooled, homogenized, and loaded 1:1 or 1:2 (homogenate:blood meal) into a subsequent blood meal that was presented to the next cohort of uninfected mosquitoes. Mosquito pools from the 10th serial passage were used for sequence and fitness assays.

Cell culture infections. BHK-21 and RML12 cells were grown in MEM or Mitsubishi and Maramorosch medium, respectively, supplemented with antibiotics and 5% FBS. Virus infections were performed at 32°C with an initial MOI of 0.1 PFU/cell and harvested 24 h (BHK) or 48 h (RML12) PI.

Vertebrate Replication and Mosquito Infectivity Comparisons. Cohorts of 3–6 mice or hamsters were inoculated with comparable titers of parental or 10th passage VEEV from alternating or serial passages. To eliminate possible phenotypic effects of cell type-specific glycosylation, VEEV from 1st passage alternating and serial mouse and mosquito lineages were also used in comparison experiments. For comparisons of alternating passages or serial passages with parents, the ultimate host in the alternating passage series was the same as that of the comparison strain. Each animal was bled at 12, 24, and 48 h PI, and mean viremia profiles for groups were calculated. For mosquito infectivity comparisons, blood meals were loaded with similar titers of parent or passaged VEEV and fed to groups of uninfected *A. aegypti*, and infection and dissemination rates in individual mosquitoes were calculated after 10 days of extrinsic incubation.

Generation of Marked Surrogate Parent VEEV Strain 3908. For strain 3908, we also used an infectious cDNA clone (27) to perform competition fitness assays. By modifying methodologies from Holland *et al.* (33) and Weaver *et al.* (19), the relative fitness of different viral populations was compared via direct competition of two genetically defined viral populations under identical conditions. We performed site-directed mutagenesis of the parental 3908 infectious clone to produce a synonymous mutation that ablated the *BspE1* restriction endonuclease recognition sequence (5' T \downarrow CCGGA3' \rightarrow 5' GCCGGA3') at nucleotide positions 8345–8350 of the capsid gene. The parental clone was used as PCR template with a wild-type positive sense and a negative sense mutagenesis primer. The amplicon product was digested with *SpeI* and *AflIII* and purified for insertion into the 3908 backbone that had been treated with *AflIII* and partially digested with *SpeI*.

Resulting bacterial clones were screened to detect plasmids that did not cut with *BspE1* and plasmid DNA was then sequenced for confirmation. To verify neutrality of the *BspE1* ablation mutant (3908*BspE1*-mut), three 3908 parent/3908*BspE1*-mut mixed passages in BHK and RML12 cells and a single passage in *A. aegypti* and hamsters ($n = 3$ per virus) were performed (data not shown). To confirm stability of the *BspE1* ablation (data not shown), 3908*BspE1*-mut was passed three times alone in BHK and RML12 cells and once in hamsters and *A. aegypti*.

Competition Fitness Assays. Alternate or serial passaged VEEV 3908 was mixed with 3908*BspE1*-mut in defined ratios and used for hamster ($n = 3$) or cell culture inoculation (three independent replicates). Twenty-four hours PI in hamsters or 48 h PI for BHK and RML12 cells, RNA was extracted from sera and amplified by RT-PCR, using primers flanking the capsid *BspE1* restriction site. ≈ 500 ng of amplicons were purified by eluting the DNA fragments from 1% agarose gels and digested with *BspE1* (NEB) for 2 h or overnight at 37°C. Intensity values of parental DNA (uncut 358-nt fragment) to passaged (257- and 101-nt cut fragments) viruses were quantified in ethidium bromide-stained bands in 1% agarose gels with densitometry (Scanalytics) and corrected for size differences and expected heteroduplexes as described in ref. 17; controls representing both unmarked and marked virus RNA were included on each gel.

Sequencing. Viral RNA from alternating or serial passages was extracted from serum or mosquito pools, using a RNA extraction kit (Qiagen) or TRIzol LS (Gibco). RT-PCRs were performed by using the Titan One Tube RT-PCR kit (Roche). Primer sequences are available upon request. Complete genomes, excluding the 5' 20 highly conserved nucleotides corresponding to the plus strand primer, were generated by sequencing PCR amplicons with the ABI Big Dye Terminator v3.1 kit and 3100 Sequencer (Applied Biosystems).

Statistical Analyses. To detect the significance of between-means viremia titers for direct replication comparisons and to compare proportions of infected mosquitoes, repeated measures ANOVA tests and Fisher's exact tests were performed, respectively. To compare starting and final ratios in competition assays, Student's *t* tests were performed.

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