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Evolutionary Influences in Arboviral Disease

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Abstract Arthropod-borne viruses (arboviruses) generally require horizontal transmission by arthropod vectors among vertebrate hosts for their natural maintenance. This requirement for alternate replication in disparate hosts places unusual evolutionary constraints on these viruses, which have probably limited the evolution of

arboviruses to only a few families of RNA viruses (*Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Rhabdoviridae*, *Reoviridae*, and *Orthomyxoviridae*) and a single DNA virus. Phylogenetic studies have suggested the dominance of purifying selection in the evolution of arboviruses, consistent with constraints imposed by differing replication environments and requirements in arthropod and vertebrate hosts. Molecular genetic studies of alphaviruses and flaviviruses have also identified several mutations that effect differentially the replication in vertebrate and mosquito cells, consistent with the view that arboviruses must adopt compromise fitness characteristics for each host. More recently, evidence of positive selection has also been obtained from these studies. However, experimental model systems employing arthropod and vertebrate cell cultures have yielded conflicting conclusions on the effect of alternating host infections, with host specialization inconsistently resulting in fitness gains or losses in the bypassed host cells. Further studies using in vivo systems to study experimental arbovirus evolution are critical to understanding and predicting disease emergence, which often results from virus adaptation to new vectors or amplification hosts. Reverse genetic technologies that are now available for most arbovirus groups should be exploited to test assumptions and hypotheses derived from retrospective phylogenetic approaches.

1
Evolution and Systematics of the Arboviruses

Arthropod-borne viruses (arboviruses) comprise a taxonomically diverse group with similar ecology and maintenance mechanisms. Although several of these viruses can be maintained in their arthropod hosts alone via transovarial transmission and some generate persistent infection of vertebrates, most if not all of these viruses require occasional or frequent horizontal transmission among vertebrate hosts by biological vectors, in which replication must occur. Therefore, arboviruses must acquire and retain fitness for replication in disparate vertebrate and invertebrate hosts. This fundamental difference with respect to most animal RNA and nearly all animal DNA viruses, which tend to specialize, on certain taxa of vertebrates, arthropods or other animals, presents unique evolutionary challenges along with many advantages of vector transmission such as high mobility and the lack of a need to be shed into bodily secretions. These challenges have probably greatly influenced the evolution of vector transmission by limiting it to only a few families of RNA viruses and a single taxon of DNA viruses.

1.1
RNA Viruses as Arboviruses

The vast majority of arboviruses are classified into only a few families and genera of RNA viruses: the alphaviruses (one of two genera) in the family *Togaviridae*; the flaviviruses (one of three genera) in the family *Flaviviridae*;

the bunyaviruses, nairoviruses, and phleboviruses (three of five genera) in the family *Bunyaviridae*; the orbiviruses (one of nine genera) in the family *Reoviridae*; the vesiculoviruses (one of six genera) in the family *Rhabdoviridae*; and the thogotoviruses (one of four genera) in the family *Orthomyxoviridae*. The only DNA arbovirus known is *African swine fever virus* (*Asfarviridae*: *Asfarvirus*) (Karabatsos 1985; Calisher and Karabatsos 1988; van Regenmortel et al. 2000); this lack of DNA arboviruses suggests that the greater genetic plasticity and higher mutation rates exhibited by RNA viruses (Holland and Domingo 1998) facilitate their ability to replicate alternately in disparate vertebrate and invertebrate hosts.

Arboviruses cause a wide range of diseases in humans and domestic animals. However, there is relatively little evidence of severe disease in reservoir hosts; most of the apparent disease caused by arboviruses involves humans, equines and other ungulates, and other domestic animals representing dead-end infections that do not exert long-term evolutionary pressures. The lack of apparent disease in many reservoir hosts may reflect selection for resistance by populations exposed for long time periods to infection and/or selection for attenuation of arboviruses in these species. These competing hypotheses are difficult to evaluate experimentally aside from the use of model cell culture systems (see below). However, the recent introduction of West Nile virus into North America provides a unique opportunity to observe these hypothetical evolutionary pressures on an arbovirus in vivo, in nature (Weaver and Barrett 2004).

Retrospective evolutionary studies of two of the major groups of arboviruses, the alphaviruses and flaviviruses, and the diseases they cause are briefly reviewed below.

1.2 The Alphaviruses

The *Togaviridae* is the only virus family comprised almost exclusively of arboviruses. Aside from Rubella virus (the sole member of the genus *Rubivirus*) and two alphaviruses with no known vector (*southern elephant seal virus* and *salmon pancreas disease virus*), all togaviruses are mosquito-borne viruses in the genus *Alphavirus* (Weaver et al. 2000). In humans and domestic animals, alphaviruses cause a spectrum of disease ranging from inapparent to highly pathogenic syndromes including arthralgia accompanied by rash, and severe, often fatal encephalitis (Griffin 2001; Tsai et al. 2002). The most important causes of severe morbidity and mortality include the New World members Venezuelan (VEEV), eastern (EEEV) and western equine encephalitis virus (WEEV), etiologic agents of encephalitis in humans and equines, and Old World alphaviruses that cause a severe but self-limiting arthralgia and rash

syndrome, including Ross River, Chikungunya, and o'nyong-nyong viruses. Epidemiological studies suggest that the latter viruses can use humans as amplification hosts during some outbreaks; otherwise, the alphaviruses generally use birds or small mammals as reservoir and amplification hosts, with humans and domestic animals representing dead-end infections. However, a notable exception is VEEV, which exploits equines as highly efficient amplification hosts, resulting in explosive and widespread epidemics

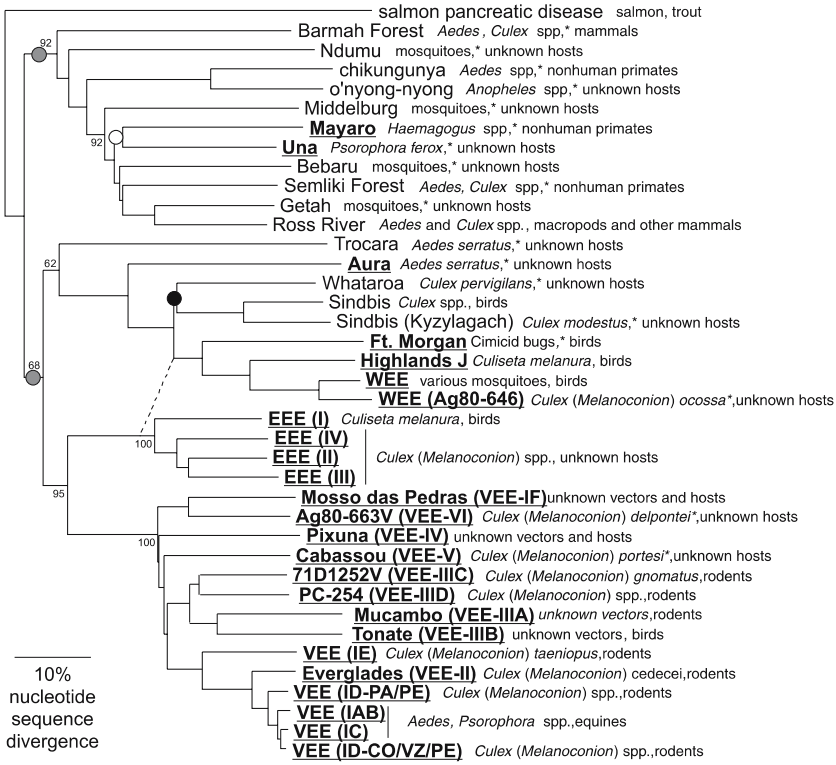

Fig. 1 Phylogenetic tree of all species and major lineages of alphaviruses derived from E1 envelope glycoprotein sequences. Subtypes are written in *parentheses* after virus names. Reservoir hosts and vectors are listed after viruses. New World viruses are printed in *bold and underlined*. *Open circle* indicates virus introductions from the Old to New Worlds, and *closed circle* indicates introductions from the New to Old Worlds; *hashed circles* indicate introductions with ambiguous directionality. *Dashed line* represents the recombination event that led to the ancestor of WEE, Highlands J and Ft. Morgan viruses. The tree was drawn using the neighbor joining program with the HKY distance formula using PAUP 4.0. Similar topologies were produced using maximum parsimony and Bayesian methods

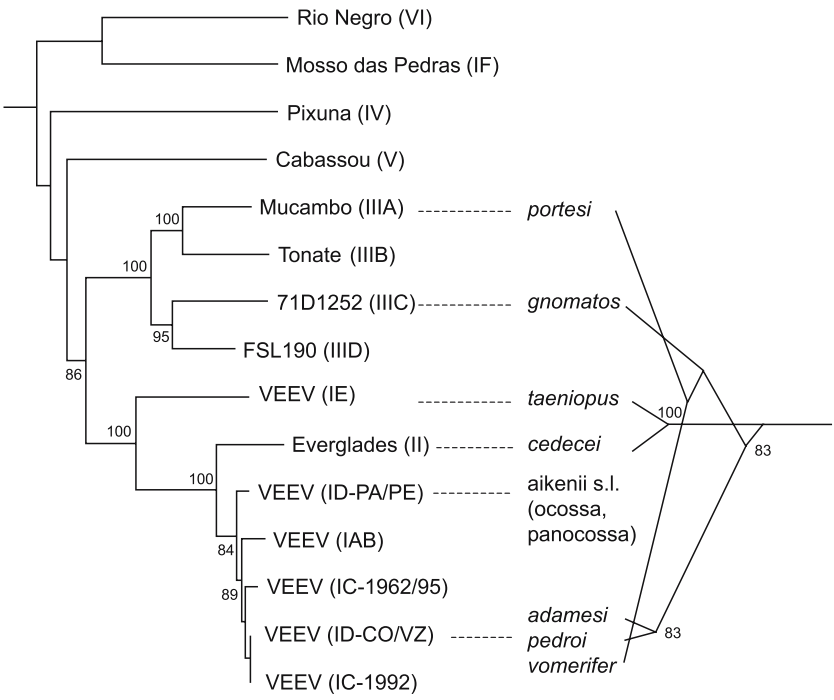

Fig. 2 Phylogenetic trees of VEE complex alphaviruses derived from structural polyprotein amino acid sequences using the neighbor joining program, and of their mosquito vectors derived from ribosomal ITS-2 DNA sequences (Navarro and Weaver 2004). Discordance in the topologies indicates a lack of co-speciation of the viruses with their enzootic mosquito vectors

(Weaver et al. 2004b). Outbreaks of VEE appear to involve adaptation of equine-avirulent, sylvatic enzootic strains for equine replication, involving small numbers of envelope glycoprotein gene mutations (Figs. 1, 2). Adaptation to new mosquito vectors, also involving envelope glycoprotein amino acid changes, appears to mediate some but not all outbreaks as well (Brault et al. 2002b, 2004; Ortiz and Weaver 2004, Brault et al. 2004).

1.2.1
Evolution of the Alphaviruses

1.2.1.1
Relationships Within the Genus

Comprehensive phylogenetic analyses of the genus alphavirus have been used to elucidate patterns of evolution and epidemiology (Powers et al. 2001).

Although Rubella virus is clearly closely related to the alphaviruses based on genome organization and functions of the major proteins, sequence divergence is extensive and cannot be demonstrated statistically aside from conserved motifs in the nonstructural proteins. Sequence analyses also have demonstrated homology among the nonstructural proteins of alphaviruses and those of several plant virus groups with dissimilar genome organizations, indicating a process of modular evolution leading to these groups (Strauss and Strauss 1994).

The alphaviruses, with no known vectors, are the most divergent members of the genus and, although rooted trees are inappropriate due to the lack of a closely related outgroup for the alphaviruses, probably represent a basal clade (Fig. 1). The distribution of these fish and seal viruses in both the Old and New Worlds provides no information on ancestral distributions to estimate the geographic origin of the mosquito-borne members of the genus.

Serocomplexes of alphaviruses first defined by antigenic cross-reactivity (Calisher and Karabatsos 1988) generally correspond to clades defined by phylogenetic studies (Fig. 1). These include the Old World Semliki Forest and New World VEE and EEE complexes. The WEE complex represents a geographically and pathologically diverse group including the new World WEEV, Ft. Morgan (FMV), and highlands J viruses (HJV) some of which cause equine and/or human encephalitis, the Sindbis-like viruses including Whataroa and Sindbis (SINV) from the New World, and Aura from the New World, which can cause a human arthralgia syndrome. The dichotomy in disease syndromes and distribution of the WEE complex viruses is most easily explained by an ancient recombination event between a SINV-like virus and the ancestor of the WEE-HJV-FMV group, followed by introduction of a descendant of the SINV ancestor into the Old World (Fig. 1; see below) (Hahn et al. 1988; Weaver et al. 1997).

1.2.1.2 Patterns of Host Utilization

Examination of host relationships in the alphavirus tree (Fig. 1) also suggests patterns of host switching and a lack of co-speciation of the viruses with their hosts and vectors. Vector species and genera vary widely within virus clades, serocomplexes and even species, with only a few exceptions: (a)the VEE complex viruses probably use exclusively members of the Spissipes section (a group of only 23 species) within the subgenus *Culex* (*Melanoconion*) as enzootic vectors. However, some if not all relationships among the vector species (Navarro and Weaver 2004) are discordant with virus relationships, indicating a lack of co-speciation (Fig. 2); (b) with the exception of the North

American strains of EEEV, all of the EEEV and VEE complex lineages also appear to use these *Culex* (*Melanoconion*) vectors, suggesting that either genetic or ecological constraints limit vector switching to closely related mosquitoes. The almost complete lack of alphavirus vectors outside of the mosquito family (*Culicidae*), including lack of evidence for an important role of ticks, which are vectors of several other arbovirus taxa, suggests similar constraints for the arboviral alphaviruses as a whole.

Alphaviruses use a wide variety of mammalian and avian vertebrate hosts for their maintenance reservoir hosts (Fig. 1). In contrast to their relationships with vectors, where a given alphavirus typically uses one or a few mosquito species as primary vectors, individual alphavirus species and lineages may use several different vertebrates simultaneously; for example, EEEV infects a variety of passeriform birds in enzootic swamp habitats of North America, many of which generate viremia sufficient for horizontal transmission by the highly susceptible and ornithophilic enzootic vector, *Culiseta melanura* (Scott and Weaver 1989). Although an important role in maintenance has not been established for many groups, alphaviruses like EEEV infect an extremely diverse group of vertebrates, including birds, mammals, amphibians, and reptiles. The wider vertebrate host range of the alphaviruses compared to the range of their hematophagous arthropod vectors suggests greater potential for reservoir than vector host switching during the course of evolution and disease emergence. Studies described below have begun to test this and related hypotheses experimentally.

The uniformity in vector taxa (mosquitoes) used by the alphaviruses is also observed in other arbovirus taxa, and contrasts with the wide range of vertebrate hosts that serve as reservoirs and amplification hosts, typically including both birds and mammals. This pattern suggests that adaptation to different vectors, such as other biting flies or ticks, is genetically difficult, and/or that arboviruses have evolved as generalists for their vertebrate hosts but specialists with respect to their vectors. However, the specificity for vectors is often manifested only at the level of midgut infection, and most alphaviruses replicate in most mosquitoes following intrathoracic inoculation, which is analogous to infection of a vertebrate via a vector bite or needle. Better understanding of the interactions between host factors and arboviruses during infection and replication is needed to understand differences in vertebrate and vector host specificity.

The taxa used as reservoir hosts appear to strongly influence the genetic structure of the alphaviral populations (Mackenzie et al. 1995; Weaver 1995). Those viruses that use avian hosts, such as EEEV (Brault et al. 1999), HJV (Cilnis et al. 1996), WEEV (Weaver et al. 1997), Barmah Forest virus (Poidinger et al. 1997), and SINV (Norder et al. 1996; Sammels et al. 1999) appear to

evolve within a small numbers of broadly distributed lineages, presumably reflecting efficient dispersal by birds. In the case of SINV, lineage replacement may occur in Australia (Mackenzie et al. 1995). In contrast, the alphaviruses that use mammals with limited dispersal, such as Ross River (Sammels et al. 1995), chikungunya (Powers et al. 2000), and most of the VEE complex viruses (Powers et al. 2001), evolve within a greater number of geographically limited lineages, reflecting very limited dispersal ability. Presumably, efficient dispersal acts to constrain lineage diversity by resulting in frequent mixing of populations and elimination of less fit populations via competition.

1.2.1.3 Rates of Evolution

Studies on the rates of sequence evolution in alphaviruses have yielded estimates that generally fall below those of single host taxon, non-arthropod-borne RNA viruses (Weaver et al. 1992). These estimates as well as analyses of sequence change obtained from phylogenies, which emphasize the preponderance of synonymous substitutions, suggest that strong purifying selection dominates alphavirus evolution. Even the 26S subgenomic promoter sequence that is conserved but includes a few differences among alphaviruses shows no evidence of adaptive substitutions, and most of the promoter sequences are interchangeable between SINV and other species (Hertz and Huang 1992). Studies of genetic diversity within alphavirus populations indicate a quasispecies distribution of genetic variants similar to that exhibited by single-host RNA viruses, suggesting that mutation frequencies are not the explanation for the genetic stability observed (Weaver et al. 1993). The requirement for alternate replication in disparate hosts is a possible explanation for this phenomenon and for the slow rate of sequence change (see below).

The time scales of evolution for the alphavirus genus as well as other arbovirus taxa have also been estimated using various genetic formulas and sequence evolution models coupled with phylogenetics. These analyses have typically yielded estimates on the order of thousands of years for divergence of arbovirus groups from common ancestors. However, the strong evidence of co-speciation of certain rodent-borne viruses with their reservoir hosts, including some in the family *Bunyaviridae*, which includes many arboviruses, indicate a time scale of tens of millions of years for these RNA virus groups (Morzunov et al. 1998). The bunyaviruses and arenaviruses exhibit genetic diversity comparable to or in some cases greater than those exhibited by arboviruses. Therefore, reconciliation of time scales derived from co-speciation evidence coupled with the fossil record, vs phylogenetic techniques, which differ by several orders of magnitude for divergence of these groups, is

problematic. One possible explanation is that the phylogenetic methods are not yet capable of accurately compensating for multiple substitutions of nucleotides and variation in the rates of substitution among nucleotide sites (Holmes 2003). Assumptions that most synonymous nucleotide sites in RNA viral genomes are subject to little or no selection, and therefore exhibit little variability in substitution rates, may be invalid due to genome-scale, ordered RNA structures that can now be identified using improved computational tools (Simmonds et al. 2004). These structures need to be examined experimentally to determine their influence on RNA virus evolution.

1.3 The Flaviviruses

The genus *Flavivirus* comprises a highly diverse group of both vector-borne and non-vector-borne viruses distributed nearly worldwide (Gould et al. 2003). Included in this taxon are important causes of human encephalitis such as Japanese (JEV) and tick-borne encephalitis viruses (TBEV), yellow fever virus (YFV), which is among the most virulent human pathogens and remains an important cause of mortality in Africa and South America, and dengue viruses (DENV), the leading arboviral causes of morbidity and mortality. In addition to their overall greater diversity compared to the alphaviruses, the flaviviruses exhibit a wider range of transmission cycles and vectors; some flaviviruses have no known vector, and large monophyletic groups use either mosquitoes or ticks as vectors (Fig. 3).

1.3.1 Relationships Within the Flavivirus Genus

The flaviviruses comprise one genus in the Family *Flaviviridae*. The other genera, *Pestivirus* and *Hepacivirus*, are non-vector-borne animal viruses. Within the genus Flaviviruses, four major clades of viruses include non-vector-borne, tick-borne, and two mosquito-borne groups. Like the alphaviruses, the flaviviruses are distributed nearly worldwide except for in Antarctica. They also infect a wide range of vertebrates and arthropod vectors, including ticks, which are not considered important vectors of alphaviruses.

1.4 Evolution of the Flaviviruses

1.4.1 Patterns of Host Utilization

Phylogenetic studies have identified interesting differences in evolutionary patterns among the four flavivirus groups mentioned above. Greater genetic

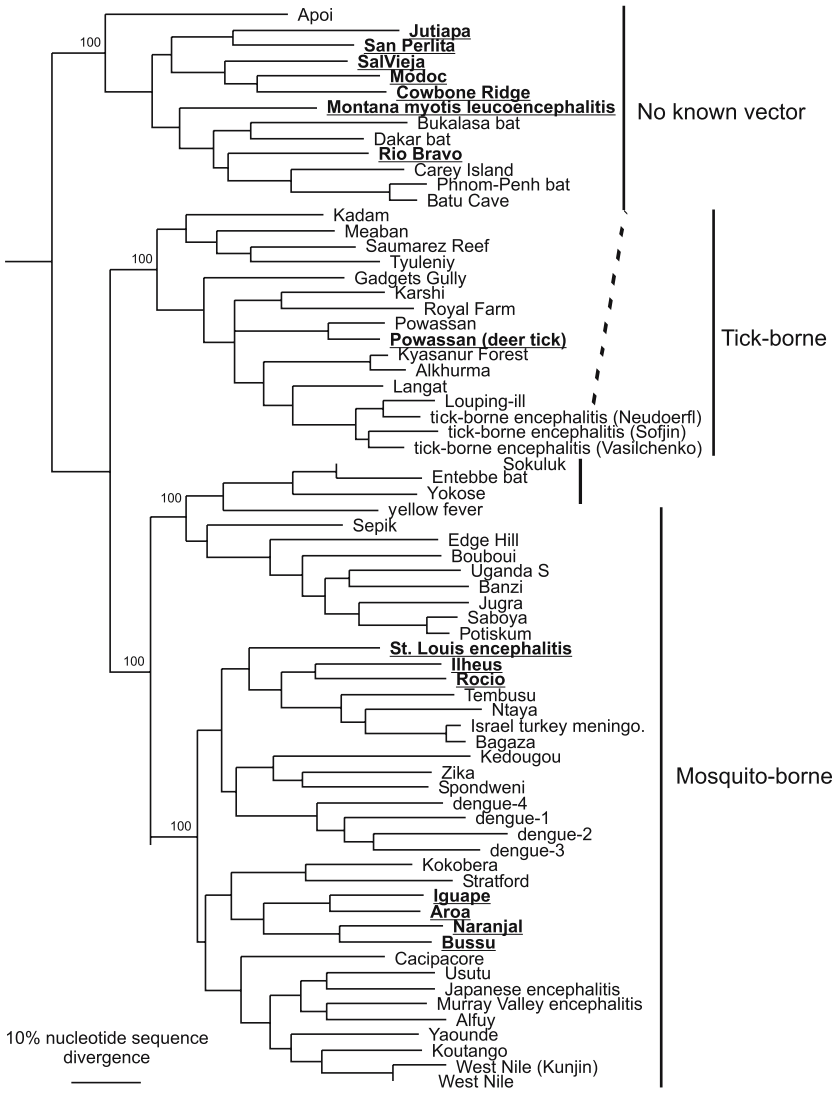

Fig. 3 Phylogenetic tree of the flaviviruses derived from partial NS5 sequences. Subtypes are written in *parentheses* after virus names. New World viruses are printed in *bold and underlined*. The tree was drawn using Bayesian methods and similar topologies were produced using maximum parsimony and neighbor joining. *Numbers* indicate bootstrap values for major clades to the *right*

conservation in the tick-borne than mosquito-borne groups has suggested that different selective constraints operate during the evolution of these two groups (Shiu et al. 1991). The tick-borne viruses appear to have evolved in a progressive, clinal pattern from east to west across Asia and Europe (Zanotto et al. 1995), while the mosquito-borne flaviviruses have evolved in a more discontinuous manner, probably in several different regions of the world. The mosquito-borne viruses tend to exhibit relatively long time periods between lineage divergence, suggesting a "boom and bust" pattern of intense diversification followed by extinction of many lineages (Zanotto et al. 1996). The best examples of this pattern are DENV, which appear to be undergoing a period of rapid radiation (Holmes and Twiddy 2003a). Detailed maximum likelihood analyses of DENV isolates to analyze rates of synonymous vs nonsynonymous substitution suggest that different genotypes or lineages experience different selective pressures, including positive selection on some amino acid sites implicated in virulence and transmissibility (Twiddy et al. 2002a). Amino acid positions subject to weak, positive selection were also identified in the envelope glycoprotein of some but not all DENV serotypes. The majority of these amino acid sites were located in, or near to, putative T or B cell epitopes, suggesting immune selection, as well as in the NS2B and NS5 genes of DENV-2 (Twiddy et al. 2002b). These kinds of studies implying positive selection should be followed up with reverse genetic validation of fitness effects in mosquito vectors or surrogate model systems for human infection.

As for the alphaviruses, the mobility of the reservoir hosts appears to have a strong influence on the population structure and evolution of flaviviruses. Those that use birds as reservoir hosts, like Japanese (Solomon et al. 2003), St. Louis (Kramer and Chandler 2001), and Murray Valley encephalitis viruses (Lobigs et al. 1988), as well as West Nile viruses (Beasley et al. 2003) evolve within broadly distributed lineages that exhibit genetic stability (Mackenzie et al. 1995). Flaviviruses with mammalian hosts exhibiting more limited dispersal, such as yellow fever virus, which uses nonhuman primate reservoir hosts, tend to be partitioned into smaller, geographically delineated populations (Bryant et al. 2003). However, the dengue viruses, which are perhaps the most mobile arboviruses due to the extensive and rapid travel behavior of human reservoir hosts, exhibit complex patterns of evolution within multiple lineages that are frequently introduced into new locations and also appear to undergo local extinctions (Holmes and Twiddy 2003; Thu et al. 2004). Genetic studies suggest that population shifts and replacements may be selected by adaptive mutations in the DENV nonstructural proteins (Bennett et al. 2003) and in cytotoxic T cell epitopes (Hughes 2001). Fitness for transmission may be responsible for some of these population changes; evidence from *Ae. aegypti* susceptibility studies suggests that an Asian genotype that has recently

colonized the New World is more infectious than the American genotype it is replacing in some locations (Armstrong and Rico-Hesse 2003). This change in the distribution of DENV genotypes has critical public health implications because the Asian genotype is more likely to cause hemorrhagic disease (Watts et al. 1999).

Analyses of flavivirus phylogenies also indicate considerable plasticity in their relationships with vertebrate hosts, and less plasticity in vector usage. Although tick- and mosquito-borne flaviviruses are occasionally isolated from mosquitoes and ticks, respectively, it appears that their principal vectors are very stable taxonomically within these groups (Gould et al. 2003). Even within the mosquito-borne clades, generic vector relations are relatively stable, with the hemorrhagic viruses mainly using *Aedes* spp. and the encephalitic members relying principally on *Culex* spp. (Fig. 3).

Of particular interest in flavivirus evolution is the presence of a large group of animal viruses with no known arthropod vectors (Fig. 3). This group appears to have diverged early during the evolution of the flavivirus genus and may represent the ancestral phenotype. Another smaller group of bat viruses comprised of Yokose, Entebbe bat, and Sokoluk viruses appears to have lost the need for vector transmission secondarily (Gould et al. 2003). These non-vector-borne flaviviruses represent an ideal system to study the effect of vector transmission on arbovirus evolution because they share basic replication strategies and genetics with the vector-borne members of the genus.

1.4.2 Rates of Evolution

Like the alphaviruses, estimates of flavivirus evolutionary rates are generally below those of single host animal RNA viruses. Also like the alphaviruses, the detection of diverse quasispecies populations within naturally infected mosquitoes and human hosts (Lin et al. 2004) suggests that mutation frequencies are comparable to those of other RNA viruses. The tick-borne viruses appear to evolve approximately two to three times more slowly than the mosquito-borne flaviviruses, probably the result of persistent infections of ticks for longer time periods than those of mosquitoes, a result of the prolonged tick life cycle (Gould et al. 2003). Nonviremic transmission of some tick-borne arboviruses (Jones et al. 1997) may result in nearly all replication occurring in the tick vector rather than the vertebrate host, compounding the effect of the tick reproductive cycle in slowing rates of sequence change.

Based on phylogenetic trees, time scale estimates for flavivirus evolution have been estimated at 5,000–10,000 years since a common ancestor (Zanotto et al. 1996). However, as explained above, these time estimates rely on

corrections for multiple substitutions of nucleotides and estimates of rate variation across nucleotide sites that may be unreliable. The recent report that flavivirus sequences are found in the DNA genomes of mosquitoes, probably the result of endogenous reverse transcriptase activity (Crochu et al. 2004), suggests a possible mechanism for arboviral sequence stability that would not be detected using phylogenetic methods. These flavivirus DNA sequences are apparently transcribed by mosquito cells, and cellular genes are generally conserved in sequence by high-fidelity DNA replication and proofreading. Therefore, recombination between these mosquito cell transcripts and RNA from an infecting flavivirus could result in restoration of ancestral viral RNA sequences via recombination.

2 Recombination and Reassortment

As described above, evidence of recombination within the alphavirus genus is limited to the WEE complex, but the possibility of recombinants between more closely related viruses or strains has received little attention. The most likely venue for an alphavirus recombination event is difficult to predict; both mosquitoes and vertebrate hosts exhibit superinfection exclusion of sequential infection by closely related alphaviruses (Karpf et al. 1997). However, exclusion is not immediate, so sequential infection of a vertebrate within a few hours by multiple mosquito bites, or sequential infection of a mosquito via multiple, partial blood meals from two different viremic hosts could result in a dual infection.

Like the alphaviruses, there is evidence of recombination from sequence and phylogenetic studies of DENV (Holmes and Twiddy 2003). Recombinant viruses as well as both parents have been detected within an infected mosquito (Craig et al. 2003). However, this recombination appears to be intraspecific (intraserotype) and there is no evidence of recombination between different flaviviruses comparable to the origins of the alphavirus WEEV as described above. The abundance of recombination in DENV may reflect the propensity for its principal vector, *Aedes aegypti*, to take multiple, partial blood meals from several different human hosts and to rely on blood as a carbohydrate nutritional source, rather than on plant nectars like most other mosquitoes (Harrington et al. 2001). Multiple feeding may increase the chances of dual infections in both mosquitoes (from biting more than one viremic human during a short time period) and in humans (from receiving multiple *Ae. aegypti* bites during a short time period due to this vector's endophilic resting and feeding behavior, and its peridomestic larval habitats.

Reassortment of gene segments has been shown to occur extensively within the family *Bunyaviridae*, and occurs efficiently in dually infected mosquitoes when the two different viruses are ingested within 2 days (Borucki et al. 1999). Reassortant bluetongue viruses can be detected in *Culicoides variipennis* that ingest two different strains within 5 days of each other, while superinfection exclusion prevents reassortment by day 7 (el Hussein et al. 1989). A recombinant Orthobunyavirus (family *Bunyaviridae*) was recently characterized from hemorrhagic fever cases during an East African epidemic. This virus, Ngari virus, a reassortant with S and L segments derived from Bunyamwera virus and an M segment from an unidentified member of the genus, demonstrates the public health importance of arbovirus reassortment (Gerrard et al. 2004).

3
Emergence Mechanisms of Arboviral Diseases

3.1
Direct Spillover

The vast majority of arboviral diseases are zoonotic, with primary, enzootic transmission cycles involving wild animals and with humans and domestic animals representing tangential or dead-end infections that do not influence the long-term evolution of the pathogen. The simplest mechanism of infection is direct "spillover," whereby enzootic transmission in the vicinity of humans or domestic animals, or the epizootic amplification of a virus due to favorable ecological conditions such as large vector populations following rainfall, lead to direct, tangential transmission (Fig. 4). This can result from a wide host range of the enzootic vector, including both reservoir hosts and humans or domestic animals, such as transmission of West Nile virus from birds to humans by the principal enzootic vector in north America, *Culex pipiens* (Turell et al. 2002). However, arboviruses such as EEEV that utilize vectors with narrow host ranges, such as *Culiseta melanura*, which feeds almost exclusively on birds, may rely on bridge vectors that bite both birds and humans for spillover. The mosquito host range of an arbovirus and the host preferences of its vector can therefore have a strong influence on arboviral disease.

3.2
Secondary Amplification

The development of domestic animals has provided some arboviruses with the opportunity to undergo secondary amplification to increase levels of circulation and the probability of spillover to humans or domestic animals.

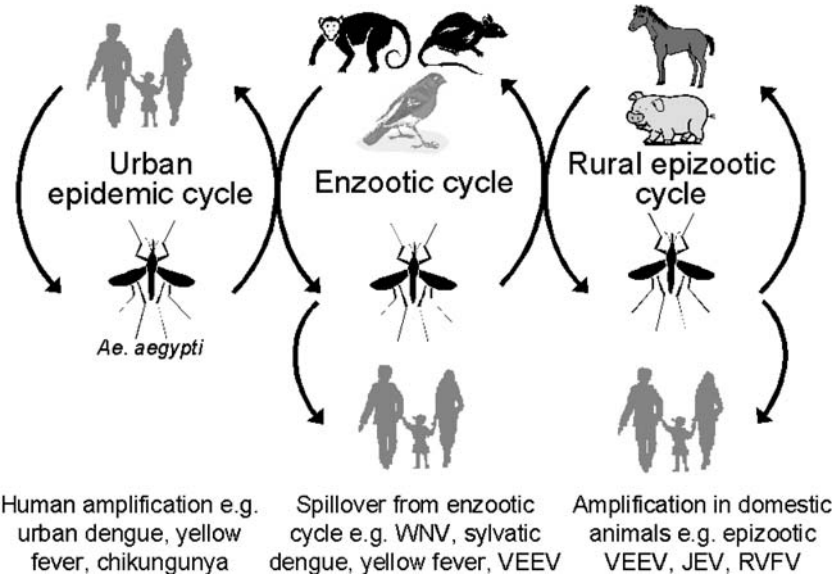

Fig. 4 Cartoon showing mechanisms of human infection by zoonotic arboviruses. At the *center* is a typical enzootic cycle involving avian, rodent, or nonhuman primates as reservoir and/or amplification hosts and mosquito vectors. Humans become infected via direct spillover when they enter enzootic habitats and/or when amplification results in high levels of circulation in their proximity. Transmission to humans may involve the enzootic vector or bridge vectors with broader host preferences including humans. At the *right*, secondary amplification involving domestic animals can increase circulation around humans, increasing their chance of infection via spillover. Examples include Rift Valley fever, Japanese and Venezuelan equine encephalitis virus (*VEEV*). In the case of VEEV, mutations that enhance equine viremia mediate secondary equine amplification. At the *left*, dengue, yellow fever, and chikungunya viruses can use humans directly for amplification, resulting in urban epidemic cycles and massive outbreaks. In the case of dengue viruses, humans also serve as reservoir hosts

Good examples include JEV, which infects pigs and chickens living in close proximity to humans in many parts of Asia, resulting in local amplification and transmission to humans by mosquitoes that do not necessarily include the avian enzootic reservoir hosts among their preferred blood sources (Endy and Nisalak 2002). Another is Rift Valley fever virus, which amplifies itself in secondary cycles involving cattle, sheep, and other ungulates (Bouloy 2001). These viruses rely on wide vertebrate host ranges, as well as on susceptible vectors that feed on several hosts, to cause human disease via spillover from secondary amplification cycles. There is no evidence that adaptation

is required for most of these secondary amplification cycles, i.e., most or all wild-type strains can readily infect these animals.

A more complex form of secondary amplification is epitomized by VEEV, the most important alphaviral pathogen of the New World. The VEEV strains that undergo sustained, continuous transmission and long-term evolution are the enzootic variants that circulate primarily in sylvatic or swamp habits, where they utilize rodents as reservoir hosts and specialize almost exclusively on vectors in the *Culex* (*Melanoconion*) subgenus (Weaver et al. 2004b). The reservoir hosts from enzootic regions generate viremia sufficient to infect the mosquito vectors yet generally develop no detectable disease. The enzootic VEEV infect people, horses, bovines, and a wide range of other hosts via spillover, with humans suffering severe febrile disease that can be fatal. Horses and bovines living near enzootic habitats become infected but develop little or no disease. The limited dispersal of the *Culex* (*Melanoconion*) vectors generally limits disease resulting from direct spillover to locations close to forest or swamp habitats (Mendez et al. 2001).

The "silent" sylvatic VEEV cycle is occasionally expanded into new habitats when mutations allow the virus to expand its host range and undergo secondary amplification, resulting in explosive equine epizootics and epidemics (Weaver et al. 2004b). Mutations in the E2 envelope glycoprotein mediate two critical adaptation events: (a) enzootic strains are selected for the generation of high titer equine viremia, which inadvertently (with respect to selection) results in equine virulence (Greene et al. 2005; Weaver et al. 2004a). Recent studies (SCW, unpublished) indicate that a single point mutation can mediate adaptation for equine viremia; (b) in some cases selection for enhanced infection of mosquito vectors that populate agricultural settings results in enhanced transmission among equine amplification hosts and humans. Adaptation to epizootic mosquito vectors can also involve as little as one mutation in the E2 protein (Brault et al. 2002a, 2004). The efficiency of VEEV in achieving dramatic host range changes with minor genetic changes epitomizes the threats naturally imposed by RNA viruses as emerging pathogens. The dramatic effect of importation of equines to the New World on VEE emergence also underscores the ability of arboviruses to exploit anthropogenic changes in unpredictable ways.

3.3 Humans as Arboviral Amplification Hosts

A few arboviruses including DENV, the most important human pathogens, have exploited host range changes to the fullest extent to cause human disease by adapting to humans as reservoir and amplification hosts. Several

arboviruses such as Ross River and chikungunya probably use humans as temporary amplification hosts during epidemic conditions, but there is no evidence that adaptation is involved. Chikungunya is particularly successful at exploiting human amplification because it uses a highly anthropophilic vector, *Ae. aegypti* (Woodall 2001). This mosquito itself underwent an evolutionary recent adaptation from the ancestral, sylvatic form found in West Africa, *Ae. aegypti formosus* (Tabachnick and Powell 1979). The derived form *Ae. aegypti aegypti* now lives in close contact with people in urban settings by relying on artificial water containers for its larval habitats, becoming endophilic to increase contact with people, and relying on blood (instead of plant carbohydrates) for its energetic needs (Harrington et al. 2001).

In many respects, DENV are the ultimate human arboviral pathogens. The ancestral forms are sylvatic strains that continue to circulate in sylvatic habitats of West Africa and Asia. These strains utilize sylvatic treehole mosquitoes as vectors and nonhuman primates as reservoir hosts (Rudnick 1984; Diallo et al. 2003). Phylogenetic studies indicate that hundreds to thousands of years ago, the four DENV serotypes each underwent ecological and host range changes to establish peridomestic and later urban transmission cycles (Wang et al. 2000; Holmes and Twiddy 2003). These endemic and epidemic DENV strains use humans as their sole reservoir hosts and peridomestic mosquitoes as vectors to cause a huge burden of human disease in the tropics and subtropics. Experimental studies indicate that the ancestral, sylvatic DENV-2 strains underwent adaptation to increase their ability to infect the peridomestic vectors, *Ae. aegypti* and *Ae. albopictus* (Moncayo et al. 2004). Adaptation to human reservoir hosts may have been equally critical to human dengue emergence. In addition, the partial cross-protectivity exhibited among the four DENV serotypes may have allowed for the co-circulation of closely related DENV strains leading to immune enhancement, which can result in severe hemorrhagic forms of disease (Ferguson et al. 1999).

A more complete understanding of the molecular determinants of host range changes responsible for the emergence of arboviruses like VEEV and DENV are critical to anticipating future disease trends and designing public health interventions. For example, several candidate DENV vaccines offer the hope of DEN eradication because humans are the only reservoir hosts for the strains circulating in most locations. However, predicting the ability of sylvatic DENV strains to re-emerge will depend on a more thorough understanding of human pathogenesis following sylvatic strain infection, and characterization of the genetic changes required to adapt to humans and peridomestic vectors. If only a small number of mutations is required, sylvatic DENV strains in Asia and Africa will represent a readily available source of new urban DENV emergence for the foreseeable future.

4
Experimental Approaches to the Study of Arbovirus Evolution

The ecological and phylogenetic approaches for studying arboviral evolution and disease emergence summarized above have revealed important host and vector associations and their evolutionary trends. They have also been used to generate mechanistic hypotheses that can be tested experimentally for optimal evaluation. For example, the slow rates of arbovirus evolution and strong evidence for purifying selection revealed by genetic studies suggest that the required alteration of vertebrate and invertebrate host infections imposed by most arbovirus transmission cycles constrains their evolution. In other words, viruses with adaptive mutations for the vertebrate host may impose fitness tradeoffs for infection and transmission by vectors, and vice versa. The identification of several alphavirus (Strauss and Strauss 1994; Schlesinger and Schlesinger 2001) and flavivirus (Lindenbach and Rice 2001) mutants with vector- or vertebrate-host-specific phenotypes and restrictions supports this hypothesis.

One approach to studying the roles of vector and vertebrate hosts on arbovirus stability has been to assess the viral genetic diversity of populations within each host. Studies of EEEV populations in naturally infected birds and mosquitoes showed no evidence of differences in genetic diversity that would assign a greater constraining selective force to either host (Weaver et al. 1993). However, greater genetic diversity has been identified in humans naturally infected with DENV-3 than in *Ae. aegypti* either naturally or experimentally infected, suggesting that the mosquito vector constrains DENV evolution (Lin et al. 2004). This constraint may simply reflect the smaller population sizes and lesser amount of viral replication in mosquitoes than in vertebrate hosts. Recent advances in nucleic acid amplification and sequencing should be exploited to further assess the heterogeneity of arbovirus populations in vertebrate and vector hosts.

4.1
Effect of the Alternating Host Cycle on Arbovirus Genetic and Phenotypic Stability

The alternating host life cycle of arboviruses and their genetic stability in nature suggest that alternating host replication may constrain adaptation, as explained above. Experimental validation of arbovirus genetic stability was first provided by studies of La Crosse virus during horizontal (oral infection of *Ae. triseriatus* mosquitoes) and vertical (transovarial transmission in mosquitoes) transmission (Baldridge et al. 1989). No RNA sequence changes were detected by RNA oligonucleotide fingerprinting in any of the passages,

corroborating genetic stability in nature. Similar studies examining transovarial transmission of Toscana virus also revealed no genetic changes during over 12 sandfly generations during a 2-year time period (Bilsel et al. 1988).

The effect of arbovirus adaptation to different hosts and cells was examined by Taylor and Marshall using the alphavirus Ross River virus (Taylor and Marshall 1975a). Serial passage in cell cultures or mice was followed by virulence testing. Passage in cell cultures depressed virulence, while mouse passage raised the level of virulence in a step-wise manner. Biological clones from both the original virus population and the 10th passage in mice were heterogeneous with respect to virulence, indicative of a quasispecies distribution. Most interesting was the finding that alternate passage between *Ae. aegypti* mosquitoes and mice resulted in no detectable change in virulence of two different wild-type virus strains (Taylor and Marshall 1975b). The authors speculated that the conservation of initial virulence by alternating mosquito-mouse passages could be related to the fact that *Ae. aegypti* can only be infected when fed on mice at the time of peak viremia, when a subpopulation of higher virulence is not present in high enough infectivity to be represented in the mosquito's blood meal (Taylor and Marshall 1975b).

4.2 Adaptation of RNA Viruses to New Hosts and Host Cells

The evolution of host range changes and host/vector alternation has been studied in several RNA viruses, only a few of which are arboviruses. Pioneering studies by Holland and colleagues with vesicular stomatitis virus (VSV) and other RNA viruses demonstrated high mutation frequencies, which allow for potentially rapid evolution (Holland et al. 1982), and the ability to rapidly adapt to new vertebrate cell lines as evidenced by dramatic increases in fitness (Holland et al. 1991). These experiments also revealed that such adaptation was often cell-specific, with fitness losses resulting in cells that were not subject to serial passages. Later, adaptation of VSV to sandfly cells was shown to reduce fitness for replication in vertebrate cells or in mouse brains, consistent with the host-specific nature of adaptation (Novella et al. 1995).

Although not an arbovirus, evolution of the RNA virus mouse hepatitis virus has been studied by Baric and colleagues (Baric et al. 1997), who attempted simultaneous adaptation of mouse hepatitis virus to mixed cell cultures containing progressively increasing concentrations of nonpermissive hamster cells and decreasing concentrations of permissive murine cells. Variant, polytropic viruses with expanded host cell ranges were generated that replicated efficiently not only in hamster and murine cells, but also in human and nonhuman primate cell lines. However, porcine and feline cells

were not efficiently infected. One derived polytropic variant was an RNA recombinant. Positive selection that appeared to be episodic occurred in the spike glycoprotein genes to allow for interspecies transfer (Baric et al. 1997).

4.3 Constraints of the Arbovirus Transmission Cycle on Adaptation to New Hosts

More direct evidence for the effect of host alteration on arboviral adaptation came from studies of VSV and alphaviruses. Llewellyn et al. showed that a natural sandfly isolate of VSV replicates more efficiently in sandfly cells than isolates of mammalian origin. When VSV was passaged alternately in sand fly and hamster cells, or allowed to specialize on one cell type through serial passages, fitness increases were observed in all cases (Novella et al. 1999). The most surprising finding was that VSV replicating exclusively in hamster cells also increased its fitness in sandfly cells, indicating that specialization did not result in cell-specific adaptation. Similar results demonstrating host range expansion following selection for replication in a single cell type have also been obtained for other, non-arthropod-borne RNA viruses (Ruiz-Jarabo et al. 2004).

The above studies with VSV suggested that arboviruses do not necessarily compromise their fitness by adapting to both vertebrate and invertebrate hosts. The number of mutations accumulated during alternated cell culture passages was similar or larger than that observed in VSV populations allowed to specialize, arguing against the hypothesis that the alternating cycle constrains rates of sequence change.

Studies with the alphavirus EEEV yielded different results and conclusions (Weaver et al. 1999). In this case, specialization on vertebrate cells resulted in fitness losses for mosquito cells, and vice versa. However, viruses forced to alternate achieved comparable fitness increases in both cell types to the specialists, contradictory to the hypothesis that alternation constrains adaptation by arboviruses. However, rates of sequence change were lower in the alternating passage series, supporting the hypothesis that host alteration constrains evolutionary rates. Similar results with EEEV were also obtained using avian and mosquito cells (Cooper and Scott 2001) and Greene et al. (in press) obtained comparable result with SINV.

Other studies with alphaviruses have focused on the subgenomic promoter and its response to selective pressure for adaptation to hosts. To determine if promoter utilization varies in vertebrate vs mosquito cells, Hertz and Huang (1995b) passaged SINV containing a library of different promoter sequences in hamster and mosquito cells. Selection was faster and more rapid in mosquito cells, which selected a smaller number of promoter sequences. Extensive

passaging of the viral libraries in hamster cells led to a promoter consensus sequence that increasingly resembled the wild type, suggesting that the wild-type and similar sequences are optimal for promoter function in hamster cells (Hertz and Huang 1995a); similar results were obtained from mosquito cell passage (Hertz and Huang 1995b). These studies suggest that SINV makes little or no evolutionary compromise in maintaining the ability to replicate alternately in the two disparate host organisms.

The inconsistencies in the results described above from different studies suggest limitations in the cell culture model systems used to study arbovirus evolution. In addition, artifactual adaptation events mediated by binding of some arboviruses to unnatural receptors such as glycosaminoglycans (Byrnes and Griffin 1998; Klimstra et al. 1998; Hilgard and Stockert 2000), which are found on the surface of both vertebrate and invertebrate cells, suggest that selection conditions in vivo might yield different results and conclusions.

In vivo studies of the effect of natural transmission cycles on arbovirus evolution are extremely limited. Preliminary studies in our laboratory have yielded results that differ dramatically from in vitro model systems. When three different alphaviruses were introduced into laboratory transmission cycles involving unnatural hosts and vectors, high degrees of genetic stability were invariably observed, with no amino acid substitutions detected following ten cycles. No fitness changes could be detected in either the mosquito or vertebrate hosts (SCW, unpublished). However, specialization of VEEV for replication in hamsters without mosquito transmission resulted in a rapid gain in fitness, with faster viremia appearance and higher peak titers (Brault 2001). These results contrast with those from the same viruses using the cell culture model systems, and are completely consistent with the hypothesis that the alternating host cycle of arboviruses constrains their evolutionary rates and ability to adapt to new hosts.

5
Future Studies

5.1
Genetic and Phenotypic Stability of Arboviruses

The genetic and phenotypic stability indicated by phylogenetic and experimental studies of arboviruses reviewed above has important public implications. As reviewed above, many experimental studies indicate the great capacity of RNA viruses to increase their virulence for vertebrate hosts, while others suggest that the alternating host transmission cycle inhibits such phe-

notypic changes. Unfortunately, several aspects of vector-borne transmission cycles have not been examined to determine their effect on arbovirus stability.

One aspect of virus transmission deserving more attention is population size, which has profound effects on the evolution of any organism (see chapters by Wilke et al. and by Escarmís et al., this volume). Large population sizes favor efficient natural selection; in the case of arboviruses, phylogenetic studies indicate that purifying selection acts to maintain phenotypic traits. However, the exact phenotypes under selection have not been determined comprehensively. The amino acid sequences of arboviral proteins are clearly one target of such purifying selection, as indicated by the overwhelming preponderance of synonymous mutations in arboviral genomes. However, in addition to conserved, *cis*-acting sequences in arboviral genomes that are under selection for primary RNA sequence and secondary RNA structure, genome-scale ordered RNA structures have been identified in some arboviruses (Simmonds et al. 2004). Such structures could introduce additional constraints on RNA virus evolution, and could also confound phylogenetic methods for estimating the ages of virus lineages due to violations of assumptions related to heterogeneity in mutation rates across nucleotide sites.

While large population sizes can suppress rapid evolution of RNA viruses, small population sizes can lead to rapid genetic and phenotypic change. In the most extreme example, genetic bottlenecks can result in inefficient natural selection and rapid genetic drift (see also the chapter by Escarmís et al., this volume). This can result in the random fixation of mutations that can be deleterious for an organism without sufficient recombinatorial capacity or opportunity, resulting in progressive fitness declines via Muller's ratchet; such effects have been demonstrated for the arboviruses VSV (Duarte et al. 1992) and EEEV (Weaver et al. 1999). Genetic drift can also facilitate the sampling of novel phenotypes, which cannot be selected in a step-wise fashion due to the lack of intermediate genotypes with improved fitness and the complexity of the selective landscape. A small population size could therefore be essential to allowing certain mutants to persist in nature. When bluetongue virus was placed in a laboratory transmission cycle involving *Culicoides sonorensis* vectors and sheep or calves, individual gene segments appeared to evolve independently by genetic drift in a host-specific fashion (Bonneau et al. 2001). In one case, a unique variant was randomly ingested by *C. sonorensis* insects that fed on a low titer blood meal, representing a genetic bottleneck, thereby fixing this new genotype by a founder effect. Additional studies of this kind are needed to assess the effects of blood meal titers on introducing bottlenecks leading to founder effects and genetic drift.

Another example of the importance of drift may be the recombinant ancestor of WEEV; sequence analyses of WEEV (Hahn et al. 1988), and adap-

tation experiments with artificially derived alphavirus recombinants (Lopez et al. 1994) indicate that adaptive mutations in the cytoplasmic tail of the E2 protein are necessary for efficient interactions with a heterologous capsid protein. Because the recombination event that generated the WEEV ancestor involved heterologous capsid and E2 genes, these results suggest that the original recombinant WEEV-like ancestor replicated inefficiently, and a population bottleneck and genetic drift are possible explanations for its initial persistence before adaptive mutations mediated more efficient replication.

These examples indicate a need to better understand the effects of the vector-borne transmission cycle on arbovirus population sizes. Unfortunately, little is known about these sizes. Viral titers in insect vectors typically reach 10^{5-7} infectious units, but the amount transmitted is usually much smaller. Estimates of mean saliva titers vary from approximately 40 to 200,000 infectious units, but mosquitoes frequently transmit far less virus (Chamberlain et al. 1954; Ross 1955; Lamotte 1960; Collins 1963; Hurlbut 1966; Gubler and Rosen 1976; Smith et al. 2005; Weaver et al. 1990; Vanlandingham et al. 2004). Viral populations within vertebrate reservoir or amplification hosts are generally very high compared to those in the vector. However, even less is known about the number of infectious virus particles that initiate the mosquito infection by entering and replicating in midgut epithelial cells. Better quantitative data on these population sizes within hosts and during transmission are needed to assess their influence on the evolution of arboviruses.

5.2 Host Switching by Arboviruses

Despite the evidence above indicating that arboviruses may be constrained in their ability to adapt to new hosts, the phylogenetic studies described above indicate that host switching is a common event during long-term arbovirus evolution. Positive selection suggested by some phylogenetic studies may reflect past adaptive events or those in progress, but for the most part have not been validated experimentally. A fundamental question requiring additional experimental studies is whether these host-switching events require adaptation or simply take advantage of pre-existing, coincidental fitness for a new host. Experiments to answer this question are now possible using reverse genetic approaches now available for many arboviruses, and are critical for predicting the emergence of future arboviral diseases. For example, many zoonotic arboviruses circulate in tropical forest habitats that are being rapidly eliminated for logging and agriculture. The elimination of these natural habitats coupled with the rapid expansion of tropical urban populations is undoubtedly placing selective pressures on arboviruses to adapt for

human-to-human transmission. The end results could be more DENV-like transmission cycles exploiting humans as reservoir and amplification hosts, with devastating public health consequences. Retrospective studies to determine the genetic determinants of adaptation to new hosts by DENV and other arboviruses will be invaluable in predicting the likelihood of additional arboviral urbanization and disease emergence.

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