Selection of generalists and specialists in viral quasispecies

Sarah D. Smith
Medical University of Ohio

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Health Science Campus

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Master of Science in Biomedical Sciences

Selection of Generalists and Specialists in Viral Quasispecies

Submitted by:
Sarah D. Smith

In partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences

Examination Committee

Major Advisor: Isabel Novella, Ph.D.

Academic Advisory Committee: R. Mark Wooten, Ph.D., Nancy H. Collins, Ph.D.

Senior Associate Dean
College of Graduate Studies
Michael S. Bisesi, Ph.D.

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Sarah D. Smith
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Abbreviations

BHK = Baby hamster kidney cells
DIP = Defective interfering particles
FDS = Frequency dependent selection
IFN = Interferon
G = Glycoprotein
L = Large Protein
Mab = Monoclonal Antibody
MARM = Monoclonal Antibody Resistant Mutant
M = Matrix Protein
MOI = Multiplicity of infection
Nt = Nucleotide
N = Nucleoprotein
P = Phosphoprotein
RdRp = RNA-dependent RNA polymerase
VSV = Vesicular Stomatitis Virus
Wt = Wild Type
**Introduction:**

RNA viruses, such as HIV, influenza, and hepatitis viruses, are major sources of human infection and disease. Although vesicular stomatitis virus (VSV) is not a major human pathogen, it serves as an excellent model for RNA virus evolution. VSV exists as complex, heterogeneous populations known as quasispecies, and undergoes frequent host changes. The high mutation rate possessed by RNA viruses allows rapid adaptation to new environments, including host immune challenges and drug treatments. In this work we tested two predictions of ecological theory that are relevant to speciation. The first prediction is that viruses replicating in homogeneous environments will become specialists, while viruses that replicate in heterogeneous environments will become generalists. Specialization would be the result of fitness costs due to differences in fitness landscapes or to tradeoffs. Results obtained in our lab show one example of fitness trade-off and two examples of costs associated with fitness landscapes. Results did not show, in contrast to previous works and predictions from ecological theory, frequent fitness trade-offs. The second prediction is that phenotypic variance in generalist populations will be higher than phenotypic variance in specialist populations. Once again, the prediction was incorrect and there was no correlation between the history or behavior of a population and its level of variation. However, populations adapting under high-MOI conditions did result in a substantial increase in variance, probably due to the ability of complementation to preserve variation. These studies contribute to the understanding of the characteristics of generalists and specialists, the process of speciation, and our understanding of quasispecies composition.
1.1 Ecological Theory

From an evolutionary standpoint, environmental heterogeneity explains the evolution of generalists and specialists. Radiation reduces competition for resources and expands genetic diversity that is ultimately beneficial for a population (Kassen and Rainey 2004). As a population replicates, variation arises. In homogenous environments, variants within populations can coexist for a period of time, but eventually higher-fitness mutants will increase in frequency, as predicted by the competitive exclusion principle, and variance will decrease as the fittest mutant dominates (Clarke, Duarte et al. 1994). The cost of specialization, which may eventually lead to speciation, occurs because specializing to one environment may come at a price of survival in other environments (Kassen 2002).

When a population replicates in heterogeneous environments, several different outcomes are possible. First, it is possible for a population to become adapted to a specific niche within that environment. When this occurs, the results are similar to those achieved in homogenous environments. Second, when a population replicates across the heterogeneous environment, rather than adapting to a niche, the population may adapt to all of the niches within the environment (Figure 1.1.1). The amount of time and the level of variation amongst niches can influence the level of adaptation (Whitlock 1992). The less time a population spends in a niche, the less it will be able to adapt, and the longer it spends in a niche, the more opportunities are presented for adaptation. Cost of generalization refers to suboptimal adaptation to each niche within the environment, such that the population is able to survive in each niche but cannot reach the level fitness
achieved by a specialist for that niche (Kassen 2002). It is often referred to with the
adage, “the jack of all trades is master of none.”

![Fitness vs Adaptation](image)

*Figure 1.1.1* A complex fitness landscape with multiple peaks is shown. Theoretically, as a population adapts to a niche, it will become a specialist for that niche. Though a population that replicates in all peaks has a wider range of resources, it cannot achieve the high fitness levels a specialist can. The dotted line represents a generalist that has adapted across the whole environment. The solid lines represent specialists that have adapted to a single niche of the environment.

The cost of specialization can have two sources: differences in fitness landscapes and tradeoffs. In the case of costs associated to the shape of fitness landscapes, a specialized population may increase in fitness in a alternate environment as it adapts to its own environment, but not as much as a population that adapts to and specializes in that alternate environment. Thus, if the two populations were to compete in the alternate environment, the specialist would win (Buckling, Wills et al. 2003) (*Figure 1.1.2*). The second source of cost is tradeoff, where replication in one environment results in fitness loss in the second environment. Tradeoffs in fitness occur in two ways: mutation
accumulation and antagonistic pleiotropy. In mutation accumulation, mutations are accrued that are neutral in one environment but are deleterious elsewhere (Levin 1968). In antagonistic pleiotropy, mutations acquired are favorable in that environment but are deleterious in another (Kawecki 1994) (Figure 1.1.3).

Figure 1.1.2 A fitness landscape with two peaks is shown. In area marked 1 we see that adaptation to environment B means co-adaptation to environment A. However, should a population that replicated in B and a population that replicated in A compete in B, the population that replicated in B would win.
1.2 Quasispecies Theory

RNA viruses have a RNA dependent RNA polymerase that lacks proof-reading abilities, allowing mutations to occur at a rate of $10^{-4}$ nucleotide substitutions per nucleotide (nt) copied per round of replication (Drake and Holland 1999). Since VSV has a genome length of 11,161nt, this averages about one mutation per genome per round of replication, allowing the formation of highly heterogeneous quasispecies with a number of variants that depart from the consensus sequence by one or more nucleotides. The formation and maintenance of a quasispecies is the result of a combination of mutation and natural selection that allows maintenance of a variety of mutants around the master sequence (the genome with the highest frequency in the population).
quasispecies can be visualized as a sort of mutant swarm or cloud. More heterogeneous populations form larger clouds, while less diverse populations form smaller clouds.

The frequency of any individual virus in the quasispecies depends on its own replication rate and the probability that it will arise by mutation of existing members of the quasispecies population. This links the members of the quasispecies, with the result that the entire population cooperates and evolves as a single structure. Therefore, selective pressures that are applied to the system are not directed to a single fit mutant, but rather whole quasispecies. This is the main factor that separates traditional mutation-selection balance theory and the quasispecies theory. VSV has been used to illustrate this concept by showing that more fit mutants are depressed by the quasispecies (de la Torre and Holland 1990). In this work, de la Torre and Holland describe a rapidly replicating clone that produced high yields of virus and is highly competitive, but fails to dominate upon continuous passages unless seeded into the population in sufficient quantities. These results indicate that quasispecies function as a group, selecting for the mutants that give the best dynamics. This study also draws attention to the idea that population dynamics may hinder the ability to correlate phenotypic effects with a single mutant from the quasispecies.

Quasispecies theory assumes that the populations replicate close to the error threshold, which is defined as “a copying fidelity value at which a sharp transition between an organized mutant spectrum and random sequences lacking information” occurs (Domingo 2001). This portion of the theory is strongly supported by experiments. In DNA microbes, mutagenesis increased the mutation frequency by several thousand fold (Cuppes and Miller 1989). In contrast, mutagenesis could only increase the
mutation frequency of VSV by three fold (Holland, Domingo et al. 1990). This indicates that RNA viruses already function near the error threshold, in contrast to DNA microbes, which do not. Additional studies with VSV show that mutagenesis did not improve virus viability or adaptability, and with increasing levels of mutagenesis, virus production was hindered in all variants tested in constant and changing host environments (Lee, Gilbertson et al. 1997).

Ribavirin, one of the most successful antivirals used to treat RNA viruses such as hepatitis C, increases the mutation rates of RNA polymerases above the error threshold leading to control of the infection. A study with poliovirus illustrates this point (Vignuzzi, Stone et al. 2005). In this experiment, poliovirus with a high fidelity polymerase was able to replicate in the presence of ribavirin. The virus replicated as efficiently as wild-type (wt), but formed a less diverse quasispecies. The restricted quasispecies was less able to adapt to stringent growth conditions. In infected animals, there was less neurotropism, and a less aggressive disease state. Mutagenic treatments that expanded the diversity of the quasispecies before infection restored neurotropism and pathogenesis, demonstrating that failure to spread to the brain was not a result of deficient replication, but of insufficient variation. When brain isolates were analyzed, evidence showed that complementation occurred between members of the quasispecies showing selection occurring at the population level.

At high viral loads in an infected host, viral quasispecies in vivo can contain every single point mutation possible, as well as additional mutants that have two or three point mutations (Nowak 2000). Maintenance of a quasispecies allows a virus access to a pool of mutants which may be more fit on a different environment (Ruiz-Jarabo, Arias et al.
In addition to the variation that is continuously generated during replication, viral populations have the ability to keep "memory variants" for several passages. Memory variants are genomes that were dominant at one point during the evolution of the quasispecies due to their higher fitness, but upon changes in the environments they decrease in frequency due to their low fitness under the new conditions (Domingo, Ruiz-Jarabo et al. 2002; Novella, Ebendick-Corpus et al. 2007). One possible mechanism that would promote the maintenance of these and other low-fitness variants is complementation (Wilke and Novella 2003). Once the environment changes again memory genomes and other previously deleterious mutants represent preexisting variation that allows for extremely rapid adaptation to new hosts.

1.3 RNA Viruses: escape mutants and vaccine design

High mutation rates promote the generation of antibody-escape and drug-resistant RNA viruses. Immune-driven evolution has been extensively studied in influenza virus. There are two mechanisms by which influenza can acquire new antigenic sites that the human immune system has not seen before: antigenic drift and antigenic shift.

Antigenic drift allows influenza A (and theoretically RNA viruses as well) to avoid detection by the immune system (Air, Laver et al. 1990). Antigenic drift occurs through accumulation of point mutations that prevent binding of antibodies induced by previous infections. As an infection progresses, the immune system and the virus play a game of cat and mouse, with the virus continually evading each new antibody the body makes. Antigenic drift is gradual and occurs over a long periods of time. Extensive evidence exists of HIV mutants evading circulating antibodies (Tomaras 2006) and
studies with VSV reveal the ability to replicate in the presence of polyclonal serum, however at a fitness cost to the virus (Novella, Gilbertson et al. 2005).

Antigenic shift occurs when entire new genes are acquired through reassortment, resulting in a large change in a very short period of time. Antigenic shift has received much attention recently, as concerns of a H5N1 influenza pandemic continue. Hemagglutinin (Hn) is the protein that enables the virus to bind to a cells surface, therefore determining the species specificity of a given strain of influenza. Neuraminidase (Nn) is an enzyme that destroys the binding between viral progeny and cell surface molecules allowing new virus particles to spread through the body. The combination of the different H and N possibilities determines the virulence of that particular strain of influenza (Oxford 2000). Invariably, the yearly influenza epidemic begins in Asia, where farmers live in close proximity with animals. Swine, which have the receptor for both avian and human influenza, become infected with both viruses, and forms a haven where the virus can re-assort, thus giving rise to a new strain of influenza each year (Peiris, Guan et al. 2001). The relevance of pig infection is most of the highly-virulent, pandemic strains of the 20th century were the results of reassortment in pigs between avian strains and human strains, resulting in new influenza variants that could infect humans but carried avian genes (Peiris, Guan et al. 2001). A more recent concern is that the influenza virus has found a way to bypass the swine intermediate and transmit directly to humans (ie H5N1). This example of antigenic shift emphasizes the importance of understanding evolutionary processes that govern host radiation and their potential for fitness trade-offs.
One of the consequences of the high adaptability of RNA viruses is the difficulty of treating viral infections, as these pathogens can produce mutants that escape antiviral drugs. For example, the ability of HIV to generate mutations conferring resistance to antiviral drugs is well known. Furthermore, the mutations are frequently present in the population even before treatment (Najera, Holguin et al. 1995; Johnson, Brun-Vezinet et al. 2007). Several examples also exist for increasing resistance to influenza vaccines (Bright, Shay et al. 2006), and thus knowledge of how RNA viruses mutate gives us an advantage in vaccine development. Simply identifying mutations that cause attenuated phenotypes is the focus of many studies that help elucidate the mechanisms by which viruses may be attenuated through evolution or by recombinant technology (Badgett, Auer et al. 2002; Tyler, Peters et al. 2007). Several studies have focused on altering virus genomes in an attempt to make less virulent viruses that can induce an immune response (Brooks, Cohen et al. 2005; Lowen, Boyd et al. 2005; Cooper, Wright et al. 2008). Many different approaches to viral vaccine development are currently being researched, with some very promising results.

Especially relevant to this work is viral attenuation through host radiation. Live-virus vaccines activate both humoral and cell-mediated immunity and generally provide longer immune protection than killed or subunit vaccines. However, live-virus vaccines must be attenuated to minimize their potential pathogenic effects. Unfortunately, the mechanisms of attenuation by adaptation to new hosts are little understood. The generally accepted method of viral attenuation has been serial passages on one or several hosts until an attenuated phenotype arises. Two examples of attenuation through passages in non-natural hosts are Sabin's oral poliovirus vaccine and Pasteur's rabies vaccine.
Historically, the selection of new hosts was based on the assumption that continued replication in any given cell line will cause attenuation of that virus. This assumption may prove dangerous, however, as shown later in this work.

The rapid adaptation of RNA viruses and the resulting quasispecies is a concern for vaccine safety and efficacy (Novella, Domingo et al. 1995). Live-attenuated vaccines are the most successful in terms of immunogenicity and protection. However, because vaccinees are infected with live virus and the inoculum replicates, there is the possibility of reversion to wt and disease development. One of the examples that has been studied in more detail is that of poliomyelitis induced by the live-attenuated oral poliovirus vaccine (OPV). The attenuated viruses used in Sabin’s OPV are capable of reverting to wt under the selective pressures of the gut within two to five weeks of vaccination in greater than 50% of people that excrete the virus (Abraham, Minor et al. 1993). This is of great concern for the immunocompromised and children who are vaccinated and are at risk of developing vaccine-associated paralytic poliomyelitis (VAPP).

Subunit and peptide vaccines are safer but have other problems. They are usually less immunogenic and the repertoire of variants that the immune response is exposed to is narrower. Thus, these types of vaccines may promote the selection of antibody-resistant mutants. One such example is that of hepatitis B virus (HBV). HBV has a DNA genome, but it replicates through RNA intermediates and, therefore, evolutionary speaking it behaves as an RNA virus. There is a safe vaccine that consists of one of the HBV proteins produced by recombinant DNA technology. Less than a decade after its introduction, escapes mutants were identified in Japan and Italy, and later in other countries (Abraham, Minor et al. 1993). Escape is usually mediated by a single amino
acid substitution within the neutralization epitope of the major S antigen (aa 124-147),
termed the 'a' determinant variants. The most commonly observed is glycine to arginine
at aa 145 (Ho, Mau et al. 1998).

In August of 2006, the CDC amended its recommendations for treating HIV to
promote use of new drug cocktails that included several kinds of drugs at the same time.
The goal was to reduce HIV loads to 50 copies per milliliter (Gulick 20006). In terms of
the quasispecies theory, the use of drug cocktails decreases the ability to generate mutants
able to escape every one of the drugs (Domingo 2001). While it is still possible to
generate such mutants through sequential accumulation of resistance mutations, these
will be debilitated due to tradeoffs (see previous section). The result is that even if there
is still virus replicating, its fitness will be too low to cause disease (Devereux, Emery et
al. 2001; Novella, Gilbertson et al. 2005). This modern application highlights the
importance of further understanding the composition of quasispecies.

1.4 Ecological theory

Ecological theory makes several predictions concerning host range and population
variance (Kassen 2002). Ecologically speaking, it is beneficial for variation to exist in a
population. If this were not true, we would see the rise and fixation of a single
phenotype. As environments change, it is necessary for populations to be able to adapt to
these changes, and even in constant environments, viruses continue to adapt to that
environment until they reach a fitness peak. Ecological theory predicts that generalists
will have a larger population variance, because different variants are required to thrive in
each of the environments that the generalist grows in. In contrast, specialists will have a
smaller population variance, because less variation is needed when replicating in a single environment, but will be less able to diversify (Buckling, Wills et al. 2003).

Several studies have examined this concept, and have lent valuable experimental data to the theory. Schneider et al (Schneider and Roossinck 2000) were the first to compare genotypic variation during infection of three alpha-like plant viruses with different host ranges. Their work indicated that these viruses had different quasispecies cloud sizes in a common host that related to the width of that virus’ host range. A follow up work showed that the quasispecies cloud size was also determined by the host, and very soon after infection variation reached maximum levels and then remained constant for several passages (Schneider and Roossinck 2001). After inoculation of the viruses on a number of hosts, they found that the quasispecies size was maintained as long as the viruses were within a specific environment. Changing hosts resulted in a change of cloud size associated with the new host. Ciota et al (Ciota, Ngo et al. 2007) completed a study with West Nile virus (WNV) which indicated that increases in the size of the mutant spectrum correlated with an increased ability of the virus to replicate in a new host. They also observed evidence that the size of the mutant spectrum may partially depend on the host, which agrees with findings by Schneider and Roossink (Schneider and Roossinck 2001).

Ferris et al (Ferris, Joyce et al. 2007) used phage Φ6 to study the effects of mutation rate on quasispecies composition and ultimately host expansion. They found that the virus was able to produce enough appropriate mutations in the P3 gene, which is implicated in host range expansion, to recognize new receptors in the surface of different host cells, indicating that mutation rates are not the limiting factor in host expansion.
Considering the constant threat of pandemic viral diseases such as H5N1 influenza virus to the human race, quasispecies size and the ability of viruses to expand to new hosts is highly significant and warrants further study.

1.5 VSV

1.5a Disease

Vesicular stomatitis virus (VSV) is a non-segmented, negative-stranded RNA virus. Its short replication time, wide host range, and high mutation rate makes it a valuable tool in the study of ecology and evolution (Novella 2003). In nature, however, VSV is an important disease of cattle, horses and pigs. VSV is a member of the family Rhabdoviridae, genus vesiculovirus. Two serotypes have been described: Indiana and New Jersey. There are three subtypes within the Indiana serotype, classic Indiana (Indiana 1), Alagoas (Indiana 2) and Cocal (Indiana 3). VSV is an arbovirus that infects livestock (cows, pigs and horses) and is transmitted by several insect species, including sand flies and black flies (Virology 2001). VSV is transmitted to mammalian hosts through an insect vector, typically through blood meals, but grasshoppers may be a vector and transmit the virus when they are eaten by cows (Nunamaker, Lockwood et al. 2003). Mammals transmit the virus to each other through respiratory droplets, direct, and indirect contact, and to the insect vector during its next blood meal. Infected vector females can transmit the virus vertically to their offspring (Tesh and Chaniotis 1975).

The clinical signs of VSV are nearly identical to those of foot-and-mouth disease (FMD) and may include vesicular lesions on the lips, tongue, gums, coronary band (area where the leg meets the hoof), snouts, and teats. Other symptoms include swelling of teats or snouts, fever, general listlessness and secondary infection of ruptured vesicles.
High titers of the virus is easily detected in the straw-colored effusion from the vesicles (Redelman, Nichol et al. 1989). Recovery from the primary VSV infection is complete around two to three weeks, but symptoms from secondary infections may last longer (Letchworth, Rodriguez et al. 1999). VSV is endemic in the Americas, and commonly causes outbreaks in these areas that can affect hundreds to thousands of animals, which can be economically devastating to local farmers who must quarantine and treat, or cull infected animals. One of the larger, more recent outbreaks in the US was estimated to cost each ranch with infected animals $15,565 (Hayek, McCluskey et al. 1998). Yearly outbreaks were reported in the US from 1900-1970, but this has reduced in the past 30 years, although outbreaks still occur sporadically (Letchworth, Rodriguez et al. 1999).

Human infection of VSV does occur, but often without symptoms. When there is disease, it is characterized by influenza-like symptoms that include fever, chills, nausea, vomiting, headache, and malaise. Human infection in endemic areas appears to be common, and is caused by direct contact with infected animals. In the 1982 epidemic in the western US, 52% of handlers working with infected animals seroconverted (Reif, Webb et al. 1987). The disease in humans is never fatal, with recovery in 3-6 days (Letchworth, Rodriguez et al. 1999). The human immune system responds quickly to VSV infection. The immune response begins with the innate response. This includes activation of interferons, complement and natural killer cells. Interferons (IFN) play a very important role in fighting viral infection. A cell that has been activated by IFN exists in an antiviral state in which over 100 genes can be activated to hinder virus replication, functions as a signal to other cells of the infection, increases the lysing
activities of NK cells, and slows the spread of the virus until the adaptive response is activated (Virology 2001). The adaptive response mediates production of antibodies that are virus specific. Antibodies neutralize the virus, most likely by blocking the G-protein spikes, however these antibodies do not provide protection against re-infection (Volk, Synder et al. 1982).

1.5b Virus Structure, Gene Products, and Replication

The VSV virion is bullet shaped, about 180nm long and 80nm wide. Its RNA genome is 11,161 nt long and consists of five genes, with a highly conserved order: 3’ N-P-M-G-L 5’.

The first protein coded for is the nucleocapsid (N) protein. The RNA sequence for the N protein is 423 amino acids long (Cortay, Gerlier et al. 2006). The main function of the N protein is to protect the virus RNA genome from RNases and other harmful factors present in host cells (Das, Chakrabarti et al. 1999). The 3-D structure of the N protein has been determined using X-ray diffraction (Green and Luo 2006). It appears that the N protein has two lobes, formed mostly of α-helices, with a valley between them in which the RNA rests. The genomic RNA winds around the N proteins, which stack on top of each other forming the basis of the helical structure that is found in the virion. Each virion contains roughly 1,258 molecules of N protein (Virology 2001). Additional functions of the N protein in replication will be discussed later.

The next protein coded for by the VSV genome is the phosphoprotein (P). It is an acidic protein of 265 amino acids (Gallione, Greene et al. 1981) and is found in various phosphorylated forms in cells. Studies that compared the sequences of many VSV genomes have determined the domain structure of VSV P protein (Bilsel, Rowe et al.
1990). The N-terminal domain is 150 amino acids and contains many phosphorylation sites. Next a highly variable region called the hinge region from amino acids 150 to 210. Residues 210 to 244 are called domain II and also contain phosphorylation sites. Finally is domain III, a basic C-terminal domain consisting of 21 amino acids. Serine and threonine residues in the N-terminal domain are phosphorylated by cellular casein kinase II (Barik and Banerjee 1992). The phosphorylated P protein forms trimers and associates with other proteins to form the active transcriptase complex (Barik and Banerjee 1991).

The VSV P gene also encodes two small proteins 55 and 65 amino acids long called C and C’ respectively. Although there is no definitive answer to the function of C and C’, though their occurrence is conserved among vesiculoviruses (Spiropoulou and Nichol 1993). A study in which a stop codon was inserted into the C and C’ coding sequences, but not the P reading frame showed that growth kinetics, viral mRNA and proteins synthesized by virus missing C and C’ was identical to wt in cell culture (Kretzschmar, Peluso et al. 1996). Thus it appears that C and C’ have a function that is not needed in cell culture, but maybe be vital in pathogenesis or transmission by insect vectors.

The third gene in the VSV genome codes for the matrix (M) protein. About 1,826 molecules of M protein are found in the assembled virus, and each protein is 229 amino acids (Gaudier, Gaudin et al. 2002). The 3-D structure was determined by crystallization (Gaudier, Gaudin et al. 2002). It is composed of a series of alpha helices and beta sheets. The interior is highly conserved among vesiculoviruses suggesting the importance of the M protein conformation. This conserved PPXY motif has been shown to effect budding (Jayakar, Murti et al. 2000). The M protein has several very important functions.
Structurally, the M protein condenses the RNA wrapped by N protein for virion assembly during budding as well as associates the N-RNA complex with the membrane during budding and in the mature virus (Chong and Rose 1993; Harty, Paragas et al. 1999; Jayakar, Murti et al. 2000). The M protein is responsible for many of the cytopathic effects seen during virus infection, including morphological changes like cell rounding (Blondel, Harmison et al. 1990; Jayakar and Whitt 2002; Kopecky and Lyles 2003; Kopecky and Lyles 2003). One way cytopathic effect is caused by the M protein is a block of export of native mRNA from the host cell nucleus (Her, Lund et al. 1997; Petersen, Her et al. 2000). By blocking mRNA production, the virus frees the cells translation machinery, allowing the virus full use of the resources the cell offers. This also effectively slows or stops cellular anti-viral defenses, as the cell cannot translate the mRNAs that code for proteins that signal other cells, such as interferon (Ferran and Lucas-Lenard 1997). In addition, the M protein has been shown to reduce cellular RNA transcription levels by inhibiting cellular RNA polymerases (Ahmed and Lyles 1998).

The fourth of the five genes in the VSV genome codes for the membrane glycoprotein (G). The rhabdovirus virions have an external membrane obtained from the infected cell. The virus surface is covered by about 400 trimeric spikes of G protein (Virology 2001). The VSV G protein consists of 511 amino acids, from which a N terminal signal sequence is cleaved forming a 500 amino acid protein (Rose and Gallione 1981). It is a type I membrane glycoprotein (spans the membrane only once) with most of the amino acids exposed on the outer virus surface. The shape of the glycoprotein is an inverted cone shape (Doms, Lamb et al. 1993) with the major carbohydrate components being mannose, galactose, N-acetylgalactosamine, and neuraminic acid
The G protein has three structural domains with the ectodomain being further divided into four domains (Roche, Bressanelli et al. 2006). Structurally, the transmembrane portion of the proteins is hydrophobic and 20 amino acids long (Reading, Penhoet et al. 1978). The C-terminal cytoplasmic domain is 29 amino acids long and contains a YXX aliphatic motif that is required for protein exit from the ER and localization to the membrane before insertion (Sevier, Weisz et al. 2000). The N-terminal ectodomain is 463 amino acids long, and provides multiple epitopes for neutralizing antibodies. The functional domains are: domain I which interacts with a cellular receptor, domain II which interacts with other G proteins to form trimers, domain III which contains the epitopes that are neutralized by antibodies, and domain IV, which is a β barrel fusion domain that allows penetration into the host cell membrane. Immediately after translation of the glycoprotein, but before disulfide bonds are formed, a chaperone called BiP associates with the protein and assists in folding (Machamer, Doms et al. 1990). After folding, the G protein forms trimers and leaves the ER. Before insertion into the membrane, G protein associates with a second chaperone calnexin (Hammond and Helenius 1994).

The final protein coded for by the VSV genome is the large (L) protein. This protein is by far the largest at 2,100 amino acids (Virology 2001). The L protein has many functions and it acts as a subunit of the viral RNA-dependent RNA polymerase (RdRp) that functions in transcription and replication. The L protein is also involved in mRNA capping, methylation and polyadenylation (Whelan, Barr et al. 2004). These functions will be discussed more in detail in the next section. The transcriptase activity
of the L-protein is stabilized and protected from proteolytic degradation by phosphorylated P protein (Gao, Greenfield et al. 1996).

1.5c Viral Replication

**Adsorption:** Viral infection begins with virus attachment to the host cell surface. The VSV cell receptor is not currently known. Although it was suggested that phosphatidylserine is the cell receptor (as binding is not inhibited by proteases, heating, neuraminidase, but is inactivated by phospholipase (Carneiro, Lapido-Loureiro et al. 2006)), subsequent studies showed that PS most likely plays a secondary role, but is not the cellular receptor itself (Coil and Miller 2004). For VSV, the optimal pH for adsorption is between 6.0 and 6.5 and optimal temperature is 23-25°C (Matlin, Reggio et al. 1982).

**Entry and Uncoating:** After adsorption, the virus is internalized by endocytosis in clathrin-coated vesicles (Matlin, Reggio et al. 1982). The vesicle’s pH is regulated by Na\(^+\) and K\(^+\) ATPases (Cain, Sipe et al. 1989) which reduce the pH which causes a structural change of the G protein allowing penetration of the vesicle and subsequent release of the ribonucleoprotein core into the host (Matlin, Reggio et al. 1982).

Studies of the pre and post-fusion forms of the G-protein suggest a pathway for fusion (Roche, Rey et al. 2007). After the virus attaches to the cell surface, it enters the cell via the endocytic pathway. As the pH changes, G adopts at least three conformational states: the native pre-fusion state at pH above 7, the active hydrophobic state (which interacts with the membrane during fusion), and the post fusion conformation. In the pre-fusion state, the fusion domains are set wide apart and have exposed hydrophobic residues. The pre-fusion state flips both the fusion domain and the
C-terminal domain to form the post-fusion form. During the transition of the fusion domains from one end of the molecule to the other, intermediates are formed. In one of the intermediates, the fusion domain extends from the top of the spike allowing it to interact with the cell membrane. Conserved histidines cluster together, and protonization of these residues at low pH likely destabilizes the interaction between the C-terminus, priming the initial movement towards the target membrane. It appears that the change in about 15 trimeric G-proteins is used to overcome the high energetic barriers encountered during fusion. A β-barrel pore unit is formed, and it is this unit that penetrates the cell membrane and forms a sort of tunnel for the RNA to enter. After penetration, in a mechanism that is not defined as of yet, the ribonuclear core is ejected into the cell cytoplasm.

**Transcription:** The components for the VSV transcriptase are carried with the virus and enter the cytoplasm upon uncoating. The transcriptase is a complex of L protein, P protein and the cellular factors GT, Hsp 60, and EF-1αβγ (Qanungo, Shaji et al. 2004). The complex enters at the 3’ end of the N open reading frame, and transcription begins followed sequentially by the rest of the genes following the start-stop model. A stop signal, 3’ AUAC(U7) 5’, between each of the genes causes a pause during which polyadenylation occurs by polymerase stuttering over the seven uracil residues to produce the poly A tail (Stillman and Whitt 1997). After polyanenylation, 70-80% of the polymerases scan through the intergenic sequence and transcribe the next gene. The remaining 20-30% dissociate from the genome, resulting in a progressive attenuation of transcription of the remaining genes (Stillman and Whitt 1997). Thus, attenuation at intergenic regions serves as a mechanism to regulate gene expression and may account
for the highly conserved gene order in rhabdoviruses and other mononegavirales 3’ N-P-M-G-L 5’ (Virology 2001). The L protein adds and methylates the 5’ cap at the guanine-M7 and 2’O-adenosine position (Testa and Banerjee 1977). The final methylated 5’ cap functions to protect mRNA from exoribonucleases, recruits the 40S ribosomal subunit and acts as the docking site for the translational proteins (O'Mullane and Eperon 1998).

**Genome Replication:** Replication differs from translation in that the polymerase complex must now ignore the stop signals and make full length positive stranded RNA from which full-length negative stranded RNA is made to be packaged in the mature virus. Replication requires concurrent transcription of N and P mRNA (Peluso and Moyer 1988). The replication polymerase complex differs slightly from the transcription complex. After transcription, the newly made N and P proteins interact with the polymerase complex, causing L to disassociate from the cellular cofactors. The N-P-L complex now has the ability to enter at the exact 3’ end of the genome, allowing replication of full-length genome (Qanungo, Shaji et al. 2004). Although several models exist for the switch between transcription and replication, the one currently accepted involves the level of N and P protein present. This model explains why transcription occurs when a virus first enters the cell and replication occurs later (when N and P levels are sufficient to switch to replication).

**Assembly and Budding:** Assembly can be divided into three main phases: (1) encapsidation of genomic RNA by N protein, (2) condensation of the RNP core by M protein at the membrane, and (3) particle release (Virology 2001). In the cytoplasm, the full length genomic RNA is associated with N, P and L proteins forming a loosely coiled ribonucleocapsid (RNP). The M protein associates with the RNP and performs the vital
function of tightly coiling the RNP to prepare it for packaging (Kaptur, Rhodes et al. 1991). After condensation of the RNP by M protein, the virus buds from the cell surface, taking with it a portion of the host cell membrane in which virus G protein is inserted (Robison and Whitt 2000).

1.6 VSV as a model for evolution

VSV has many qualities that make it suitable as a model for evolution. It has a short replication time (around 9 hours for maximum virus production), can infect many types of cells from mammalian or insect origin (Wagner 1987), can reach high titers, and has a high mutation rate. The small genomic size allows the determination of full-length sequences in evolved genomes and there are reverse genetics systems to analyze mutations individually or in combination. Furthermore, VSV has been a model for mononegavirales for many decades so its molecular biology is well characterized. These traits, the ease at which VSV can be cultivated in the laboratory, and the ability to manipulate single aspects of experimental design makes VSV an excellent model for evolution. In the last 30 years, VSV has been used to examine several relevant concepts that can have contributed to the areas of evolutionary biology, RNA virology, and viral disease treatment.

1.6a Genetic bottlenecks and Mueller’s Ratchet

Because of the high mutation rates of the RdRp, large genetic loads of deleterious mutations are expected, and indeed, examination of 98 plaques from a single clonal population found the majority of the populations had deleterious mutations (Duarte, Novella et al. 1994). Mueller (Mueller 1964) predicted that because a significant
proportion of mutations are deleterious, repeated bottleneck of asexual populations will result in the irreversible accumulation of deleterious mutations in a ratchet-like manner. In sexual reproduction, this effect can be avoided by recombination. Muller's ratchet has been described in experimental populations of many RNA viruses (Chao 1990; Clarke, Duarte et al. 1993; Duarte, Novella et al. 1994; Escarmis, Davila et al. 1996; Novella, Quer et al. 1999; Yuste, Sanchez-Palomino et al. 1999). In viral populations the most drastic of genetic bottlenecks (reduction of population size) can be produced through plaque-to-plaque transfers, thus reducing the population size during transfer to a single virus particle. Whatever mutations the randomly picked plaque(s) contain, whether beneficial or deleterious, are passed on to progeny, when they otherwise may have been out-competed by a more fit mutant.

After determining that Mueller’s Ratchet does occur in VSV (Duarte, Clarke et al. 1992), research turned to determining its effects. Clarke et al (Clarke, Duarte et al. 1993) found that five of nine populations subjected to Mueller’s Ratchet had significant decreases after only 20 bottleneck passages. The remaining four had fitness that remained neutral. However, when the same beginning populations were passaged in large population passages, they observed large fitness gains in all replicas. When the low fitness bottlenecked passages were subjected to large population passages, they regained high fitness. Additional studies found that the higher the fitness of the founding quasispecies, the more likely that population is to undergo fitness losses during low dose transmission because the majority of the mutants from that population have lower fitness (Novella, Elena et al. 1995). Mueller’s Ratchet has long-term effects, as well as the immediate, stochastic drops in fitness. Genetic bottlenecks comprimise the capacity of
viral populations to adapt, as shown by bottlenecked population’s reduced ability to regain fitness and outcompete wt in situations where the initial fitness of the bottlenecked population is the same as that of the wt (Novella 2004).

Modeling Mueller’s ratchet is important to evolutionary biology because it mimics the bottlenecks that a virus is subjected to during transmission. Respiratory droplets usually only contain one or a few infectious particles (Gerone, Couch et al. 1966). HIV sequences from newly infected individuals are homogenous compared to the more diverse quasispecies that is found in the presumed donor, indicating that HIV undergoes a bottleneck upon transmission between hosts (Pang, Shlesinger et al. 1992; Wolinsky, Wike et al. 1992). A similar situation was observed in newly infected persons with hepatitis C and in long-infected individuals (Feray, Samuel et al. 1992). The studies on Mueller’s ratchet shed light when in determining appropriate treatment in RNA virus infections. There was some controversy regarding the best timing for antiviral treatment in HIV-infected patients. The studies on Muller's ratchet indicate that the best option is to treat patient as soon as possible, when variation is low (with a low probability of having resistant mutants) and fitness is low (and therefore the virus is weakened).

1.6b Defective Interfering Particles and Complementation

Defective interfering particles (DIP) are deletion mutant viruses that have the ability to enter cells, but are missing the components needed to replicate and require co-infection with a standard virus to replicate (Holland 1991). DIPs derive their name from their ability to interfere with the replication of wt virus through the sequestration of its proteins (Holland 1991). DIPs are frequently produced at a high multiplicity of infection (MOI). MOI is the average number of viruses that infect each cell and it determines the
degree of complementation that will take place. Complementation is the process by which a virus utilizes a gene product from another virus co-infecting the same cell. In the case of DIPs this means the utilization of the products necessary for the DIP to replicate and package its defective genome (Holland 1991). DIP production is believed to occur when a viral polymerase along with its attached nascent RNA chain dissociates from its the template that it is copying and continues replication at another point on the same template or anywhere in new template (Perrault 1981). Hypermutated and defective genomes are found in several disease, including lethal human infections of measles with CNS involvement (Cattaneo and Billeter 1992), chronic HBV infections (Santantonio, Jung et al. 1991), and HIV (Meyerhans, Cheynier et al. 1989) illustrating one of the clinical aspects which VSV research can be related to.

Phenotypic mixing and hiding is a specific example of complementation involving surface proteins. The phenotype of a virus may not reflect its actual genotype, as the genotype may be encapsidated by another virus’ proteins (Novick and Szilard 1951; Holland, de la Torre et al. 1989; Valcarcel and Ortin 1989). Phenotypic mixing and hiding, as well as other types of complementation, may lead to the underestimation of mutation rates. Holland et al calculated mutation rates in VSV looking at the frequency of monoclonal antibody resistant mutants in plaque assays. Mixing virus stock and antibody before plating resulted in mutation frequencies of $2 \times 10^{-5}$, one order of magnitude less than actual mutation rates. This low value was obtained because when mab was added before penetration, monoclonal antibody resistant (MARM) genomes with overwhelmingly more abundant wt surface proteins were neutralized, and therefore unable to propagate. By changing the method to add mab to the overlay after viral
penetration, all genomes were able to enter cells, but only those with a MARM genome were able to form plaques, thus preventing phenotypic mixing and hiding. As a result, the mutant frequencies observed by this method were up to 1,000-fold higher (Holland, de la Torre et al. 1989). Phenotypic mixing and hiding has been suggested as a contributing agent to quasispecies memory (Wilke and Novella 2003). Several studies have found low fitness mutants that are maintained for long periods of time in the quasispecies population, even though their low fitness should cause their extinction (Domingo, Ruiz-Jarabo et al. 2002; Novella, Ebendick-Corpus et al. 2007).

1.6c Red Queen Hypothesis, Critical Points, and Competitive Exclusion

The Red Queen Hypothesis refers to a situation in which two competing populations must evolve, just to remain in stable competition with each other (Van Valen 1973). The term “Red Queen” refers to a character in Lewis Carroll’s book *Through the Looking Glass*, who says to Alice, “It takes all the running you can do to keep in the same place.” Clarke et al (Clarke, Duarte et al. 1994) conducted a very interesting set of experiments with VSV that show this phenomenon. Here, neutral MARM clones were mixed with wt and allowed to compete. The two populations coexisted for many passages, but eventually one population out-competed the other. When the fitness of winners and losers was determined the results showed that all the populations had increased their fitness, but the winners increased their fitness more than the losers. This result also provides experimental support to another principle of population biology known as the competitive exclusion principle, which states that when two populations compete for the same limited resources, one population will always exclude the other (Gause 1971). Clark et al (Clarke, Duarte et al. 1994) noted that the exclusion of the
losing population was always abrupt and decisive. It appeared that since both populations were continuously increasing in fitness, only largely superior mutants were able to exclude the other population.

In a subsequent publication, Quer et al (Quer, Huerta et al. 1996) described a mutant (MARM C) which was initially neutral but was always out-competed by wt. Furthermore, the kinetics of MARM C decay were surprisingly predictable and led to the identification of critical points, which were defined as “points from which viral competitions may follow different trajectories.” In this experiment Quer et al (Quer, Huerta et al. 1996) used the same mixture of MARM:wt to initiate six long term passages. All six followed similar trajectories until a critical point, when MARM started decreasing in relation to wt in a very reproducible and predictable manner. These two experiments were mathematically modeled later by Solé et al (Sole, Ferrer et al. 1999) who determined that the similarities between MARMs and wt implies symmetry between the populations which allows a period of similar evolution in accordance with the Red Queen Hypothesis. Quer et al (Quer, Huerta et al. 1996), however showed that although MARM C and wt had equal starting fitness, the history of MARM C involved a series of bottlenecks that destroyed some of the symmetry between the systems, thus allowing its consistent demise when competed long term against wt.

1.6d Frequency Dependent Selection

An exception to the competitive exclusion principle can occur in frequency dependent selection, where it is possible to find a frequency at which two populations may stably and indefinitely coexist (Ayala 1971) if fitness changes in relation to frequency. Frequency dependent selection (FDS) has been shown to occur in many

![Figure 1.6d.1](image)

**Figure 1.6d.1** Schematic representation of the three possible relationships between fitness functions $W_A$ and $W_B$ (Forster and Wilke 2007). In case I, $W_B$ is superior, in case II, neither is superior (and thus can coexist at equilibrium) and in case III $W_A$ is superior.

It is noted, however, that the equilibrium observed need not be stable, they can cycle, or have even more complex interactions.

While frequency dependent selection has many forms, one common type is known as “fittest when rare.” One example of this is the afore-mentioned DIPs and complementation. When DIPs are rare, they are able to coinfect with wt, thus utilizing wt’s resources. However, the more DIPs are produced (shorter genomes mean shorter replication times), the more resources are stolen from wt, leaving wt with less gene products for its own replication, higher numbers of DIPs, more chances for coinfection of two DIPs, and no wt to complement the defective genome.
Chapter 2: Materials and Methods

2.1 Revisiting the Cost of Host Radiation

The virus, cells and methods for the host radiation experiments are identical to those described by Turner and Elena (Turner and Elena 2000) unless otherwise indicated.

Viruses and Cells. Two genotypes of VSV Indiana serotype, Mudd-Summers strain were used: I1 monoclonal antibody-sensitive wild type, and MARM U, an I1 monoclonal antibody resistant clone. MARM U has a single nucleotide substitution at nt 3853 that confers resistance to I1 without altering the fitness (except in the presence of I1 Mab). For experimental passages and plaque assays, BHK-21 cells were used. HeLa and MDCK cells are from the European cell culture collection. HeLa cells are human epithelial cervical carcinoma cells that are highly characterized (Gey 1952). MDCK cells are Madin-Darby canine kidney cells. MDCK and HeLa cells were grown as monolayers in Eagle’s minimal essential medium (MEM) containing Earl’s salts and 10% fetal bovine serum (FBS) and were incubated in 95% humidity and 5% CO₂. BHK-21 cells are baby hamster kidney cells, and were obtained from John Holland’s lab. They were grown in MEM containing Hanks salts and supplemented with 7% heat inactivated bovine calf serum (BCS) and 0.6% proteose peptone No. 3. Cell monolayers were all grown at 37°C and were passaged about twice each week by trypsinizing cells off the walls of the cell culture flask and transferring a portion of the cells to a new culture flask. Overnight monolayers are prepared in the same way.

Experimental Passages. MARM U was used to initiate four replicas each (labeled A-D) of four experimental regimes: 25 passages in either MDCK or HeLa cells, which served as novel cell hosts, 25 passages in BHK-21 cells, which are typically used to propagate
VSV, and alternating between MDCK and HeLa for 25 passages. Passages were performed by taking a sample of supernatant from a previous infection, diluting the sample to ensure a proper MOI, and transferring the diluted sample to a new monolayer. Two rounds of replication occur every 24 hours, and each round of replication requires at minimum one round of copying to generate antigenomes and a second round of copying to generate genomes (e.g. two generations). Thus, after 25 passages the final population was the $100^{th}$ generation. For alternating passages, the virus was passaged for 48 hours in MDCK cells, then for 48 hours in HeLa cells, and so forth. In our experimental design we continued alternating passages an additional 25 passages (for a total of 50 alternating passages or 25 cycles between cell types).

For viral passages, virus were first diluted and $2 \times 10^5$ PFU were used to infect confluent monolayers with 2-3 million cells grown on the surface of a T-25 tissue culture flasks. Thus, the MOI was approximately 0.01 PFU/cell. Each cell monolayer was infected with 200μL of the virus solution. A 10-minute incubation at room temperature allowed the virus to attach to the cells, followed by a 40-minute incubation at 37°C to allow penetration to occur. Finally, 5mL of MEM medium supplemented with FBS was added to the monolayer, which was then allowed to incubate to allow viral replication. Infections were allowed for 24 hours (BHK-21 infections) or 48 hours (MDCK and HeLa infections) at 37°C and the progeny was diluted $10^{-4}$ to carry out the next passage. Diluting the population for the next infection ensured that the MOI remained low, thus preventing DIPs from interfering. The final passage was collected and stored at -80°C for further analysis.
All the populations were tested for I1 Mab sensitivity to determine whether they had retained the genetic marker used for fitness determinations. Sensitivity was assayed by triplicate plaque assays in the presence and absence of monoclonal antibody (Mab) I1; plaques were counted and the averages were compared using a t-test.

**Fitness determinations.** Fitness assays were carried out by direct competition between Mab I1-resistant, evolved virus and Mab I1-sensitive, reference wild type in HeLa, MDCK and BHK-21 cells. Fitness was calculated as R1/R0, and each fitness value was the average of at least two independent determinations.

Fitness assays were preformed making a mixture of two viruses, in this case evolved MARM and wt (P0). The mixture was then diluted so competitions were started at MOI of 0.01. Again, 200µL of the solution was applied to a BHK monolayer, incubated at RT for 10 minutes, 37°C for 40 minutes and then 24 hours at 37°C after the addition of 5mL MEM medium supplemented with BCS. Additionally, P0 was diluted and plaque assays were infected to calculate the true begining ratio of wt:MARM. During the 24-hour incubation, the two viruses compete for the resources in the flask. The virus that is able to produce the most progeny (i.e has the highest fitness) will increase in frequency during the competitions.

A sample of each competition passage was collected and diluted to obtain 50-250 PFU in each flask, because these are numbers that are easily counted in plaque assays and provide the best statistical power. For each plaque assay, a monolayer was infected with 200µL of virus solution, incubated at RT for 10 minutes, 37°C for 40 minutes and then 24 hours at 37°C. This time however, the medium was solidified with agarose, which only allows virus progeny to infect neighboring cells, forming distinct plaques. For
competition assays, duplicate sets of monolayers were infected, one set received normal media, and the other received media supplemented with I1 Mab. After the 24 hour incubation, the agarose overlay was removed and the monolayer was fixed and stained with a mixture of ethanol and crystal violet. The cells infected by the virus detach from the flask, leaving holes (plaques) where the virus was replicating. These plaques were counted and a ratio of MARM (which grows in the presence of I1) and wt (which is calculated from the plaques counted from the monolayer without I1 Mab) was determined.

As mentioned previously (see section 1.1, 1.6c) populations adapted to a given environment usually show a fitness increase in that environment. Because we expected very high fitness values in some of the adapted populations, and since the fitness assay method has limitations in the amount of excess MARM U that can be reliably measured, instead of pre-adapting the reference virus for HeLa and MDCK competitions, we tested for frequency-dependent selection. We carried out this analysis by first competing selected populations from each regime at initial ratios between 1:1 and 40:1 (see section 3.1). Because frequency-dependent selection was not observed, we completed the fitness assays, adjusting the initial wt:MARM ratio as needed. Using excess wt levels the playing field in the competition assay, allowing the wt to be detected even though it produces less progeny than the adapted MARM.

2.2 Evolution of variance in RNA virus quasispecies

In order to analyze the level of variance observed in viruses with different evolutionary histories, multiple replicas of each population were tested using fitness assays. Ecological theory predicts that populations with a history of passages in single
environments should behave as specialists and have low variance. In contrast, populations with a history of passages in heterogeneous environments should behave as generalists and have high variance. We chose 16 populations to carry out this analysis.

**Cells and viruses**

Cells used in this experiment were LL-5 sandfly cells (derived from *Lutzomyia longipalpis* (Tesh and Modi 1983), BHK-21 cells from John Holland’s lab, and HeLa cells and MDCK cells from the European cell culture collection. Acute BHK infections take place over 24 hour periods, however, acute infections of LL-5 cells last 48 hours and infections are incubated at 28° C. VSV does not cause cytopathic effect in LL-5 cells and generates about $10^8$ PFU/ml during that period versus the $10^{10}$ PFU/ml that BHK cells produce.

Our control was wt (or MARM U) prior to passages. The remaining 15 populations had a history of replication in single cell types or alternating cell types and their origin is outlined in table 2.2.1. Included in this table are the expectations of each quasispecies variance as predicted by ecological theory.

- MDCK, HeLa and passages alternating between the two (MH50 and MH25) have been described in the previous section. MDCK and HeLa adapted virus have a history that would make them specialists, while virus alternating between HeLa and MDCK has the history of a generalist.
- Wt virus used as a control is Indiana serotype, and has been well characterized. It is not classified as a specialist or generalist.
- A25 and B25 replicas are virus that has adapted during 25 acute passages in BHK cells. KA80 and KB80 are direct descendents of A25 and B25.
Serial passages were simply carried on for additional passages. A80 and C80 are additional wt passages on BHK cells, however, they are not descendents of A25 and B25, but are separate replicas. These replicas exhibit exponential increases in fitness as they adapt to the cell host and have histories that classify them as specialists.

- L80B and L80D are serial acute passages in LL-5 cells (Novella, Hershey et al. 1999). Though sandfly cells become persistently infected with VSV, they release virus into the supernatant. To force acute infections, a sample of the supernatant was used to infect a new LL-5 monolayer. L80B and L80D showed overall fitness increases. These viruses are classified as specialists according to their history.

- KL80B was formed by serial passages alternating between BHK and LL-5 cells for 80 passages (Novella, Hershey et al. 1999). These replicas have a general exponential increase in fitness, but it is much more erratic by nature. Their adaptation history classifies them as generalists.

- Per is a population that has been persistently infected and A25B alternated between persistent LL-5 infection and acute BHK infection. As these populations adapted, they both saw an increase in fitness of about three orders of magnitude, and then remained approximately constant (Zarate and Novella 2004). According to their adaptation histories, Per is a specialist and A25 is a generalist.

Fitness assays were carried out as described for the fitness trade-off experiment, however, 2000 PFUs of test virus and appropriate amounts of control (wt or MARM U)
were competed rather than the $2 \times 10^5$ PFUs normally used in competitions. Ideally we would have analyzed individual clones from each population by selection individual plaques for analysis, but this was not possible due to the presence of very tiny, pinprick plaques in some of the populations. These plaques are very difficult to see even after staining, and there was a high probability that plaque picking would be biased towards the larger plaques, leading to misleading results. Whenever possible, two replicas of the twenty sets was performed to confirm results. To analyze the data, the average fitness was determined, and statistical variance as well as the coefficient of variation was calculated using the statistical program Kaleidograph.
Table 2.2.1 Virus History and prediction of quasispecies variance. The virus name and passage history are outlined in the first two columns. The third column indicates the level of variance predicted by evolutionary theory based on the passage history of each population. ↑ indicates some increase in variance, ↑↑ indicates large increase in variance, ↓ indicates some decrease in variance, and ↓↓ indicates a large decrease in variance.

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Wild type virus. Reference strain with fitness of 1</td>
<td>→</td>
</tr>
<tr>
<td>A25/B25</td>
<td>WT after 25 passages on BHK cells</td>
<td>↓</td>
</tr>
<tr>
<td>A80/C80</td>
<td>WT after 80 passages on BHK cells</td>
<td>↓↓</td>
</tr>
<tr>
<td>KA80/KB80</td>
<td>WT after 80 acute passages on BHK cells</td>
<td>↓↓</td>
</tr>
<tr>
<td>L80B/L80D</td>
<td>WT after 80 acute passages on sandfly cells</td>
<td>↓</td>
</tr>
<tr>
<td>HeLaC</td>
<td>MARM U after 25 passages on HeLa cells</td>
<td>↓</td>
</tr>
<tr>
<td>MDCKC</td>
<td>MARM U after 25 passages on MDCK cells</td>
<td>↓</td>
</tr>
<tr>
<td>MHC25</td>
<td>MARM U alternating between HeLa and MDCK, 25 passages</td>
<td>↑</td>
</tr>
<tr>
<td>MHC50</td>
<td>MARM U alternating between HeLa and MDCK, 50 passages</td>
<td>↑↑</td>
</tr>
<tr>
<td>KL80B</td>
<td>WT alternating acute infections between sandfly and BHK cells</td>
<td>↑↑</td>
</tr>
<tr>
<td>Per</td>
<td>WT after 80 persistent passages</td>
<td>↓</td>
</tr>
<tr>
<td>A25B</td>
<td>WT alternating between persistent sandfly infection and acute BHK infection</td>
<td>↑</td>
</tr>
</tbody>
</table>

Chapter 3: Results and Discussion

3.1 Cost of Host Radiation

To confirm that the founding MARM U population and the resulting adapted populations are not polymorphic at the nucleotide that confers antibody resistance, we completed sensitivity assays for all populations (data not shown). Using both a one-tailed and a two-tailed t-test, we confirmed that there was no statistical difference between the populations incubated with and without I1 monoclonal antibody (p > 0.13). This
indicates that both the founding and resulting populations are monoclonal antibody resistant and therefore the fitness assays correctly assess viral fitness.

Consistent with Turner and Elena’s (Turner and Elena 2000) results, we found that high levels of adaptation hindered the ability to measure accurately wt:MARM ratios after competition with initial ratios set at 1:1. The high fitness achieved by the adapted virus exceeded the detection limit of the fitness assay when we started the competitions at a 1:1 ratio. To measure fitness we determine ratios by counting plaque numbers produced in the presence and absence of I1 Mab, and then compare the ratio of wt:MARM before and after competition. Counts in the presence of I1 Mab gives the number of MARM virus, and in the absence of I1 both wt and MARM produce plaques. We then calculate the amount of wt as the difference between total virus (produced in the absence of I1 Mab) and MARM virus (produced in the presence of I1). Thus, when MARM is in substantial excess, the plaque numbers with and without I1 are similar, and the fraction that represents wt cannot be calculated with any accuracy. Turner and Elena (Turner and Elena 2000) circumvented this problem by pre-adapting wt reference virus to the two new environments (HeLa and MCDK cells). The increased fitness of pre-adapted wt allowed detection after competition against evolved MARM genotypes, but assumes that one can calculate the fitness of two viruses based on the relative fitness of each one in relation to a third virus (the pre-adapted reference). An additional problem was uncovered during the determination of genomic changes in evolved MARM populations, and that was the identification of polymorphisms in the marker loci in several populations (Remold, Rambaut et al. 2008). While in some cases the populations gained a different resistance marker, in other cases the polymorphism resulted in populations that were
partially sensitive to Mab I1. The loss of the genetic marker could result in two types of problems. First, the calculations of wt:MARM ratios, both before and after competition, are misleading, making accurate calculation of R0/R1 impossible. Second, the dynamics during competition between reference sensitive wt, evolved sensitive wt and evolved resistant MARM would be unpredictable. In this report, we reassessed the extent and direction of fitness costs and tradeoffs using standard reference virus and fully-resistant evolved populations.

To avoid the problem of MARM excess after competition, fitness assays may be started with excess of wt, so its frequency was still sufficiently high after competition for accurate ratio determinations. However, this method has another underlying assumption: that there is no frequency-dependent selection. Frequency-dependent selection is uncommon but possible in regimes of acute infection in VSV (Elena 1997; Forster and Wilke 2007). Thus, we carried out competitions at different initial ratios of wt:MARM and looked for changes in fitness as the ratio of wt to MARM changed. Our results showed no correlation to the initial ratio during competition (Table 3.1.1). With this result, we ruled out frequency-dependent selection, and fitness assays were carried out at appropriate ratios of wt:MARM.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>A on HeLa</th>
<th>A on MDCK</th>
<th>H on HeLa</th>
<th>H on M</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:1</td>
<td>ND</td>
<td>ND</td>
<td>25.5±3.5</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>10:1</td>
<td>39.3±24.7</td>
<td>13.9±0.9</td>
<td>38.3±4.65</td>
<td>ND</td>
</tr>
<tr>
<td>20:1</td>
<td>24±3.5</td>
<td>42.8±17.4</td>
<td>ND</td>
<td>0.18±0.03</td>
</tr>
<tr>
<td>40:1</td>
<td>ND</td>
<td>26.75±5.45</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 3.1.1 Test for Frequency Dependent Selection

<table>
<thead>
<tr>
<th>Virus fitness at different ratios on HeLa and MDCK cells</th>
<th>A on HeLa</th>
<th>A on MDCK</th>
<th>H on HeLa</th>
<th>H on M</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:1</td>
<td>ND</td>
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<td>25.5±3.5</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>10:1</td>
<td>39.3±24.7</td>
<td>13.9±0.9</td>
<td>38.3±4.65</td>
<td>ND</td>
</tr>
<tr>
<td>20:1</td>
<td>24±3.5</td>
<td>42.8±17.4</td>
<td>ND</td>
<td>0.18±0.03</td>
</tr>
<tr>
<td>40:1</td>
<td>ND</td>
<td>26.75±5.45</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Passaging MARM U on a host for 25 passages allowed the virus to adapt to that environment as shown by its significant fitness gains on that environment (p < 0.05 t-test) (Figures 3.1.1 and 3.1.2). HeLa-adapted virus increased fitness in HeLa cells, MDCK-adapted virus increased fitness in MDCK cells, and BHK-adapted virus increased fitness in BHK cells; these results are consistent with previous work (Novella, Duarte et al. 1995). Alternating environments at both passage 25 and 50 led to a significant increase in viral fitness on all cell lines, as predicted by theory and in accordance with previous work (p < 0.05 t-test) (Novella, Hershey et al. 1999; Weaver, Brault et al. 1999). Also interesting to note is that an additional 25 passages of virus cycling between MDCK and HeLa cells did not increase the fitness of that virus on MDCK (p < 0.001 ANOVA), indicating that by passage 25, the virus had already reached the top of its fitness peak (Figure 1). Alternating virus at passage 50 and 25 did not differ significantly when competed on HeLa cells (p = 0.59, ANOVA). Additionally, as observed in the previous study (Turner and Elena 2000), adaptation to HeLa and MDCK environment was similar (p = 0.55, t-test).
Figure 3.1.1. Fitness changes during adaptation of VSV to novel host cells (MDCK and HeLa). The data obtained in the present report are shown gray, and the data reported previously (Turner and Elena 2000) in white. Columns show fitness of evolved virus on HeLa cells (H), MDCK cells (M), and alternating cell types (A) for 25 and 50 passages. Bars indicate standard errors. ND = not determined.
Figure 3.1.2. Fitness changes in the original host cells (BHK-21) during adaptation of VSV to novel host cells. The novel host cells are MDCK and HeLa, and fitness changes during adaptation to the original host cell (BHK-21) are also included. The data obtained in the present report are shown in gray, and the data reported previously (Turner and Elena 2000) in white.

Trade-offs and fitness costs in these three cell types were less common than previously reported (Turner and Elena 2000). The only cost that we identified was in virus adapted to HeLa cells (Figure 1). HeLa-adapted virus showed an overall decrease in fitness on MDCK cells that was only marginally significant (p = 0.1, ANOVA). HeLa-adapted population also exhibited a cost due to differences in landscapes in BHK cells. HeLa-adapted populations had a slight but significant fitness increase (1.2 ± 0.19; p =
0.01, t-test), but this gain was lower than the fitness increase identified in BHK-adapted virus (W=3.8±0.4) on BHK cells (p = 0.03, t-test). Because HeLa-adapted virus did not gain as much fitness as BHK-adapted virus, this is a cost attributed to differences in fitness landscape rather than a tradeoff. In contrast, there were no costs observed in MDCK-adapted virus compared to BHK-adapted virus when fitness was determined in BHK-21 cells (p = 0.5, ANOVA) or HeLa-adapted virus in HeLa cells (p = 0.13, ANOVA). These results are inconsistent with the previous report (Turner and Elena 2000), which showed a tradeoff in fitness of HeLa-adapted virus in BHK-21 and MDCK cells, even though the authors indicate that, as a group, the fitness loss in MDCK was not statistically significant.

The majority of the evolved populations in our study incurred in no fitness costs associated with replication in homogeneous environments and behaved as generalists rather than as specialists. BHK-adapted virus increased in fitness in both MDCK and HeLa cells (p < 0.02, t-test) (data not shown). Alternating viruses did not have lower fitness in any cell line; their fitness increase in BHK-21 cells was not as dramatic as that observed in the novel HeLa- and MDCK-cell environments, but is similar to that achieved by BHK-adapted virus (p = 0.54 and 0.49 at passage 50 and 25, respectively, Two Way ANOVA), probably due to the previous history of replication in and adaptation to BHK-cells in the original MARM U population.

Several interesting points in RNA virus evolution are illustrated with this simple experiment. Previous works demonstrated that during large population passages, population fitness increases on that environment (Novella, Duarte et al. 1995; Chen, Wu et al. 2003; Novella, Zarate et al. 2004). This increase in fitness is seen here as virus
adapted to HeLa has a fitness increase on HeLa and MDCK adapted virus has an increased fitness on MDCK. The question explored in this study was whether this increase in fitness would come at a cost to fitness in other environments. The trade-off in fitness observed in replica C of virus adapted to HeLa cells in MDCK and BHK-21 cells, demonstrates a trade-off associated with specialization. Indeed, the mutations that increased fitness in a HeLa environment were detrimental to virus fitness in an MDCK or BHK-21 environment. Previous articles have pointed out that perhaps fitness trade-offs are overrated as the driving cause for specialization (Fry 1996), and ask if fitness costs due to differences in landscape profiles are too often overlooked. Experimental work with *Drosophila* (Weber 1996; Bolnick 2001), bacteria (Rainey and Travisano 1998; Sander, Springer et al. 2002; Buckling, Wills et al. 2003; MacLean, Bell et al. 2004), and viruses (Novella, Clarke et al. 1995; Feuer, Boone et al. 1999; Novella, Hershey et al. 1999; Cooper and Scott 2001; Ciota, Lovelace et al. 2007), as well as observations of natural occurrences of diverged species (Schliewen, Rassmann et al. 2001; McKinnon 2002) have led to the identification of specialization as the potential cause of speciation, yet very few works have specifically addressed costs due to landscape differences, or fail to correctly identify them, leaving a void in the knowledge of which mechanism contributes more to speciation. In this study, we showed that HeLa adapted virus did not lose fitness on BHK cells, but rather failed to increase in fitness, illustrating a cost associated with fitness landscapes. Our own group failed to report previous examples found in studies where we analyzed VSV adaptation mammalian (BHK-21) and insect (sand fly LL-5 cells). Virus adapted to replication in BHK-21 cells showed fitness increases in LL-5
sand fly cells, but those increases were lower than fitness increases observed in virus adapted to LL-5 cells (Novella, Hershey et al. 1999; Zarate and Novella 2004).

Other studies using cell culture to analyze the evolution of other RNA viruses have produced evidence of cell-specific adaptation as well as evidence for adaptation across environments. Tradeoffs during adaptation to insect and mammalian cells have been reported in Sindbis virus (Greene, Wang et al. 2005), eastern equine encephalitis (Weaver, Brault et al. 1999; Cooper and Scott 2001), and Venezuelan equine encephalitis virus (Coffey, Vasilakis et al. 2008), while experiments with VSV showed no trade-offs in fitness between mammalian and invertebrate cell lines (Novella, Hershey et al. 1999). In contrast, the cost associated with differences in landscape and the mechanism(s) of such cost are more difficult to predict. Studies with West Nile virus and St. Louis equine encephalitis virus showed a trade-off between invertebrate-adapted virus in mammalian cells, and what appears to be a cost associated with landscapes when the invertebrate-adapted virus was tested in avian cells (Ciota, Lovelace et al. 2007). The same studies also showed the cost of generalization. This cost was inconsistent and only clear in Sindbis virus (Cuevas, Elena et al. 2002) and perhaps eastern equine encephalitis virus (Cooper and Scott 2001). Research with VSV show that generalists and specialists have similar fitness values (Novella, Hershey et al. 1999; Weaver, Brault et al. 1999; Turner and Elena 2000). Interestingly, these same in vitro results were not seen in vivo when eastern equine encephalitis virus was passaged in mice and mosquitoes; the generalist in this experiment did not gain fitness on either vertebrate or invertebrate cells (Coffey, Vasilakis et al. 2008).
Comparison of previous results (Turner and Elena 2000; Remold, Rambaut et al. 2008) with those reported here allows dissecting the causes of the discrepancies. Both pre-adaptation of wt and the loss of marker in some of the population altered the results. Among the evolved populations previously analyzed (Turner and Elena 2000), those adapted to HeLa cells had retained their genetic marker and were fully resistant to I1 Mab (Remold, Rambaut et al. 2008). This result is consistent with our determinations of sensitivity of the same strains by triplicate plaque assay in the presence and absence of I1 Mab (not shown). Using pre-adapted wt for the analysis of these HeLa-adapted strains led to overall underestimation of fitness in all environments, and to the incorrect identification of tradeoffs in BHK-21 cells. Populations adapted to heterogeneous environments for 25 passages were partially sensitive to I1 Mab (Remold, Rambaut et al. 2008), also in agreement with our own analysis of those strains (not shown). In this case there was also overall underestimation of fitness values, but to a much larger degree. In addition, instead of the substantial fitness costs previously reported, we found no costs during competition of alternating viruses in BHK-21 cells. Because the competitions in BHK-21 cells were done in both laboratories using standard wt, the differences can be assigned to the loss of genetic marker. These results indicated that both pre-adaptation of the wt reference and the loss of genetic marker contributed to the discrepancies found between the previous and the current datasets.

The loss of genetic markers and the pre-adaptation of reference wt may not be the only factors contributing to differences in the results obtained in the two laboratories. Laboratory dependence (variable performance of organisms between laboratories) is unavoidable, and while we took every possible precaution to match the conditions of our
work to those followed by Turner and Elena (Turner and Elena 2000) there are things that we cannot control, such as the source of water or the batch of serum used in the growth medium. These minor differences are likely to contribute to some extent to differences in the results. Indeed, O’Keefe and colleagues (O’Keefe, Morales et al. 2006) identified deionized water as the cause of laboratory-dependent differences and quantified the discrepancies in *E. coli* fitness determination carried out in two different laboratories. However, inconsistencies due to water source were always below 10%, while here we report differences of about one order of magnitude, suggesting that the contribution of differences in laboratory procedures would be relatively small.

Remold et al (Remold, Rambaut et al. 2008) sought to identify the molecular mechanisms of tradeoffs through the determination of the complete genomic sequences in evolved populations. Looking at the distribution and frequency of mutations, they correctly concluded that the tradeoffs observed when HeLa-adapted viruses were tested in MDCK cells were due to antagonistic pleiotropy. There were six new alleles in HeLa-adapted viruses that were not found in alternating virus. If these new alleles had no cost in MDCK cells, at least one would probably been found in the alternating environment. Remold et al (Remold, Rambaut et al. 2008) also note that because the HeLa-adapted virus and alternating virus have similar increases in fitness (a result supported by our data), there is more than one way to adapt to HeLa cells. However, the mutations found in MDCK-adapted populations were interpreted as deleterious in HeLa cells due to the apparent tradeoffs previously reported. However, we have shown here that MDCK-adapted viruses did not lose fitness in HeLa cells and, in fact, they increase in fitness to an extent similar to that observed in HeLa-adapted populations. Thus, there is no tradeoff
and this tradeoff cannot be the result of mutation accumulation. Most likely, the mutations observed in MDCK-adapted virus increase fitness in MDCK, and at least some of them may also have a beneficial effect in HeLa cells. The simplest arguments that explain their absence in HeLa-adapted populations are the small number of strains sequenced and lack of enough time to allow frequency increases for those mutations during adaptation to HeLa cells.

This study highlights some of the problems that may arise during fitness analysis in the laboratory and here we propose means to address these problems. Marker loss can be avoided by periodic addition of I1 Mab during the experimental evolution of MARM populations (Clarke, Duarte et al. 1993). This method has the disadvantage that potential effects of this site on evolution would be missed. For the marker in nt 3853 this is an unlikely problem because it has been shown as neutral in a large number of environments (Novella, Duarte et al. 1995; Novella, Elena et al. 1995; Quer, Huerta et al. 1996; Novella 2004). However, resistance due to substitutions in other loci, such as at 3846 is frequently observed after adaptation to different environments – even though always in combination with a substitution at nt 2151 (Cuevas, Elena et al. 2002; Remold, Rambaut et al. 2008) – suggesting that it may sometimes have a beneficial effect and should be avoided as a genetic marker. We have found that populations evolving in BHK-21 cells always remain fully resistant to I1, even after hundreds of generations (unpublished results), but in other environments sensitivity to I1 Mab should be carefully assessed before analysis. If partial sensitivity develops, genetic analysis would still be useful, but fitness analyses should not be performed. Regarding the analysis of very high fitness MARM populations, there is no reason to do the competitions at an initial 1:1 ratio.
Changing initial ratios and adding excess of wt in the competition mixture is better than using pre-adapted reference wt. The only caveat is the potential existence of frequency-dependent selection, which is infrequent in experimental regimes where the passages are done at low multiplicity of infection (MOI) and can be tested doing an initial set of competitions at various wt:MARM ratios. Furthermore, even if there is frequency-dependent selection, there is no reason why an initial 1:1 ratio would solve any problem. The one example of frequency-dependent selection during low MOI passages in VSV (Elena 1997) showed neutrality of the test virus when the wt:MARM ratio was 0.43:1.

3.2. Changes in variance during the evolution of specialists and generalists.

Ecological theory predicts that the variance of a populations will correlate with its history, so viruses replicating in constant environments (single cell types) will behave as specialists with low phenotypic variance, and genotypic variance and viruses replicating in heterogeneous environments (alternating cell types) will behave as generalists with high variance.

In general, fitness variation conformed to normal distributions. As shown in the first part of this thesis, the prediction of selection of specialists and generalists in homogeneous or heterogeneous environments was shown to be incorrect. In this section, the second part of the prediction was tested and multiple determinations of fitness were carried out for the populations described in Table 2.2.1.

The results of phenotypic distributions obtained for each population are shown in Figure 3.2.1.
Comparison of standard deviations showed a direct correlation with the average fitness of a population (Figure 3.2.2).

To avoid bias in the comparison due to differences in fitness, the coefficient of variation (CV) can be used. CV is calculated as \( c_v = \frac{\sigma}{\mu} \) and is defined as the normalized...
dispersion of a probability distribution. Higher CVs indicate more variance in a population. The results of CV determination for each population are shown in Figure 3.3.3.

Figure 3.3.2 Correlation Between Fitness and Standard Deviation. Mutants with high fitnesses have higher standard deviations.
Figure 3.2.3 Coefficient of variation of VSV populations with different backgrounds. This figure shows the CV for populations tested. White bars represent populations evolving in homogenous environments, while gray bars represent populations evolving in heterogeneous environments. WT is the control and is represented in black.

Table 3.2.1 shows the comparison between the anticipated CVs based on the phenotype of each population and the actual results (note that Table 2.2.1 in section 2.2 presents the prediction of variance changes based on the history of each population).


<table>
<thead>
<tr>
<th>Specialist</th>
<th>Avg fitness</th>
<th>Expected CV</th>
<th>CV</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.88</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A25B</td>
<td>0.44</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per</td>
<td>0.05</td>
<td>1.38</td>
<td>↑↑</td>
<td></td>
</tr>
<tr>
<td>Hela C 25</td>
<td>0.80</td>
<td>0.78</td>
<td>↑↑</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Generalists</th>
<th>Avg fitness</th>
<th>Expected CV</th>
<th>CV</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.88</td>
<td>0.39</td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>A25</td>
<td>2.84</td>
<td>0.21</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>B25</td>
<td>4.56</td>
<td>0.31</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>KA80</td>
<td>5.99</td>
<td>0.41</td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>KB80</td>
<td>5.35</td>
<td>0.40</td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>A80</td>
<td>4.82</td>
<td>0.32</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>C80</td>
<td>2.52</td>
<td>0.23</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>MDCK C 25</td>
<td>2.54</td>
<td>0.70</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>MH50 C</td>
<td>2.70</td>
<td>0.64</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>MH25 C</td>
<td>2.15</td>
<td>0.64</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>L80B</td>
<td>2.02</td>
<td>0.48</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>L80D</td>
<td>1.82</td>
<td>0.46</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>KLB80</td>
<td>3.02</td>
<td>0.68</td>
<td>↑</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2.1 Expected and actual variation. Shown in this table are the tested viruses average fitness in BHK cells, their expected CV based on their behavior (specialists or generalists), observed CV and observed increase or decrease in variance.

The data showed no correlation between variance and history of passages (homogeneous vs. heterogeneous environments) or behavior of the population (specialist vs. generalist) (Table 3.2.1). It was expected that the generalist populations would have more variance, but this is not true. All the populations under analysis behave as
generalists except HeLaC25, Per and A25B, and their CV varied between 0.21 and 0.70 (compared to 0.39 in wt). The specialist populations have CV values that were sometimes higher and sometimes lower than that of wt. Interestingly, the two higher CV values corresponded to two specialists: HeLaC25 (0.78) and Per (1.38). The overall conclusion is that ecological theory relevant to host radiation does not describe well the behavior of RNA viruses, and the specific selective pressures that different cell lines impose is more important than general environmental patterns. In this respect, the high CV observed in Per has a reasonable biological explanation. Per is a strain that has a history of 25 passages of persistent evolution. Under these conditions, viral infections take place at high MOI, which results in weakening of selection and survival of low fitness mutants (see section 1.6b). Note that A25B, which differs from Per in its periodic replication in BHK cells at low MOI, has a much lower CV. This is reasonable because deleterious variation would have been eliminated during these low MOI passages. Thus, the level of coinfection is the best correlate with the level of variation.

It would be interesting to note, however, if these findings can be applied to all viruses or to a more complex system. As noted before, VSV is a quintessential generalist, infecting every mammalian cell type it is introduced to. This is not true for all RNA viruses, much less all viruses. Perhaps findings would be different with a virus that was not by nature a generalist. Also as noted before, we are using VSV as a model to test a single aspect: host cell adaptation. Perhaps in a more complex environment with more than one selective pressure, results would also be different.
Chapter 4: Summary

We have tested ecological theory predictions regarding the selection of specialists and generalists, and the evolution of variance. Results show that costs due differences in landscapes are more common than costs due to tradeoffs, and the contributions of the former to radiation, and potentially speciation, may be more significant than currently thought. We have also shown that predictions made by ecological theory are incorrect. Fitness trade-offs do not always occur, and there is no correlation between a generalist or specialist background and variation.
References:


Abstract:

RNA viruses, such as HIV, influenza, and hepatitis viruses, are major sources of human infection and disease. Although vesicular stomatitis virus (VSV) is not a major human pathogen, it serves as an excellent model for RNA virus evolution. In this work, I used VSV to test two predictions of ecological theory that are relevant to speciation. The first prediction is that viruses replicating in homogeneous environments will become specialists as a result of fitness tradeoffs or costs due to differences in fitness landscapes, while viruses that replicate in heterogeneous environments will become generalists. Results show one example of fitness trade-off and two examples of costs associated with fitness landscapes. In contrast to previous works and predictions from ecological theory, results did not show frequent fitness trade-offs. The second prediction is that phenotypic variance in generalist populations will be higher than phenotypic variance in specialist populations. Once again, the prediction was incorrect, and there was no correlation between the history or behavior of a population and its level of variation. However, populations adapting under high-MOI conditions did result in a substantial increase in variance, probably due to the ability of complementation to preserve variation.