SEARCHING FOR THE ADVANTAGES OF VIRUS SEX [*]

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Abstract. Sex (genetic exchange) is a nearly universal phenomenon in biological populations. But this is surprising given the costs associated with sex. For example, sex tends to break apart co-adapted genes, and sex causes a female to inefficiently contribute only half the genes to her offspring. Why then did sex evolve? One famous model poses that sex evolved to combat Muller's ratchet, the mutational load that accrues when harmful mutations drift to high frequencies in populations of small size. In contrast, the Fisher-Muller Hypothesis predicts that sex evolved to promote genetic variation that speeds adaptation in novel environments. Sexual mechanisms occur in viruses, which feature high rates of deleterious mutation and frequent exposure to novel or changing environments. Thus, confirmation of one or both hypotheses would shed light on the selective advantages of virus sex. Experimental evolution has been used to test these classic models in the RNA bacteriophage $\phi 6$, a virus that experiences sex via reassortment of its chromosomal segments. Empirical data suggest that sex might have originated in $\phi 6$ to assist in purging deleterious mutations from the genome. However, results do not support the idea that sex evolved because it provides beneficial variation in novel environments. Rather, experiments show that too much sex can be bad for $\phi 6$; promiscuity allows selfish viruses to evolve and spread their inferior genes to subsequent generations. Here I discuss various explanations for the evolution of segmentation in RNA viruses, and the added cost of sex when large numbers of viruses co-infect the same cell.

Keywords: experimental evolution, genetic exchange, microbe, RNA, sex, virus

1. Introduction

To most people, evolutionary biology conjures the image of a paleontologist carefully unearthing fossils, and piecing these together to create a glimpse of life in Earth's distant past. But this traditional method is impractical for reconstructing the evolutionary history of microbes such as bacteria, because their microfossils are scarce and difficult to distinguish from non-biological sources (Brasier *et al.*, 2002). The situation is even more troublesome for viruses, obligate intracellular parasites that rely on a host cell's metabolism to complete their life cycle. Viruses are exceedingly minute, and they do not feature the highly-conserved metabolic genes that often prove useful in constructing evolutionary phylogenies in bacteria and macro-organisms. Efforts to produce accurate phylogenies in viruses are further complicated because virus genomes are short in length and extremely labile.

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For instance, viruses can sometimes migrate in and out of the host chromosome, donating or accepting genetic material in the process. This can dull the distinctions between virus and host genes, and blur the search for unique virus characters in the deep past. How does one effectively search for the origin of virus traits?

Experimental evolution is a powerful method for examining the evolution of traits in the present, in order to gauge hypotheses regarding their past origins. To that end, experimental evolution concerns laboratory studies of 'evolution-in-action' (Bennett, 2002). Often this involves a single genotype (the ancestor) that is used to found populations that are allowed to evolve in replicated laboratory environments. Although the laboratory habitats are strictly controlled, evolution proceeds via natural selection because the environment determines which genotypes contribute their genes to subsequent generations. This is a very different process from artificial selection (such as cattle breeding) where the experimenter explicitly chooses which individuals contribute genes to the descendants. Microbes (bacteria, viruses) have proven to be useful subjects for experimental evolution due to their large population sizes, short generation times, and ease with which their environments and genetic systems can be manipulated. More importantly, microbes can be stored in a freezer for indefinite periods of time, permitting direct comparisons between an ancestral genotype and its evolved descendants. Here I review several recent studies where experimental evolution is used to examine the advantage of sex in viruses.

2. The Ubiquity of Sex in Nature

The biological definition of sex is any exchange of genetic material between individuals. Accordingly, sexual mechanisms are found to be extremely common in nature. Sexual reproduction is the rule in humans, and by far the majority of vertebrates. But sexual processes are also widespread in other macro-organisms, and are readily observed in microbes. For instance, bacteria experience sex through the processes of conjugation, transformation, and transduction. The consequence of sex in any biological system is the production of genetic variability, which (along with *de novo* mutations) is the raw material for evolution by natural selection. But evolution is typically a slow process, involving the change in the genetic makeup of a population or species over the course of long periods of time (measured in units of generations). One can easily observe that sexual reproduction in humans contributes to vast phenotypic differences among individuals along a crowded street. However, our generation time is long (25–30 yr), and selection pressures strong enough to change gene frequencies in a single human generation are very unusual. Thus the influences of sex-derived variation on gene frequency changes are particularly difficult to observe in our species. In contrast, the short generation times of microbes can reveal the profound significance of sex in shaping evolved traits. For example, bacterial conjugation allows unidirectional passage of DNA from
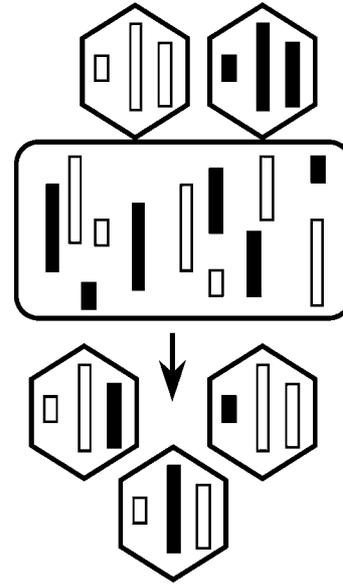
RECOMBINATION
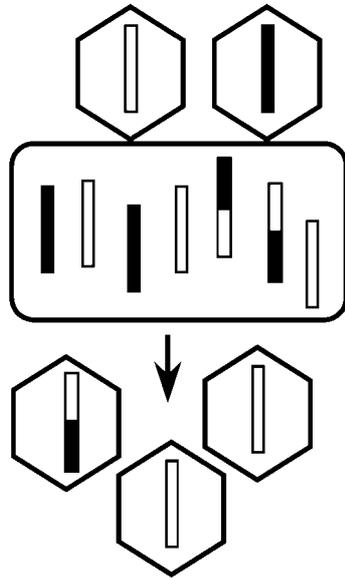
REASSORTMENT

Figure 1. Recombination can occur in both DNA and RNA viruses, whereas segment reassortment occurs only in certain RNA viruses. Virus particles: hexagons; host cells: rectangles; genetic material (DNA or RNA): black and white bars.

donor to recipient cells. Conjugation enables the rapid spread of beneficial genes in bacterial populations, and has facilitated the proliferation of resistant genotypes following widespread use of antibiotics in the middle part of the twentieth century. We are currently unable to produce novel antibiotics fast enough to stem the tide of resistant bacteria that threaten human health, demonstrating that genetic exchange in microbes can profoundly influence the ecology of other species in the ecosystem.

What are the mechanisms that permit genetic exchange in viruses? Viruses feature genomes that are composed of either DNA or RNA. In DNA viruses sex is promoted exclusively by recombination (generation of a new nucleotide strand from two or more parental strands), the same mechanism underlying sexual processes in all DNA organisms (Figure 1). In some RNA viruses sex is also by recombination, but in others it is not. Rather, genetic exchange is achieved by segmenting the viral genome into several smaller RNA molecules and hybrid progeny are produced as random reassortments of segments descending from the coinfecting parents (Chao, 1992) (Figure 1). An analogy is the shuffling of two (or more) hands of playing cards to create a series of novel hands. The genetic variation that is created through reassortment appears to facilitate the epidemiological spread of certain pathogenic RNA viruses that infect humans, such as influenza and rotavirus (Basler *et al.*, 2001; Iturriza-Gomara *et al.*, 2001). Recombination between

homologous segments is rare or non-existent in most RNA viruses that feature reassortment (Kilbourne, 1979; Tyler and Fields, 1986). Therefore, it has been suggested that reassortment evolved in these viruses as an alternative to recombination for the purpose of promoting sex (Pressing and Reanney, 1984; Chao, 1988).

The widespread occurrence of sex in nature is surprising given that sex has several easily-identified costs. From a genetic standpoint, sex is an inefficient process in organisms with male and female gametes; a female that reproduces sexually only contributes one-half of her genes to her progeny. Thus, a mutation that causes females to produce only daughters, but has no other effect, will initially double in frequency in each generation. This is often termed the two-fold cost of sex, or needless production of males (Maynard Smith, 1978). But in any biological system, another consequence of sex is that it tends to break apart well-adapted combinations of genes. Thus, whenever favorable combinations of genes are brought together into single individuals, sex has the potential to immediately tear them apart at its next occurrence (Shields, 1988). These obvious difficulties have inspired evolutionary biologists to struggle for over a century to answer the question: Why did sex evolve?

3.   Models for the Advantage of Sex

A variety of theories exists for the evolutionary significance of sex (Maynard Smith, 1978; Michod and Levin, 1988; Kondrashov, 1993; Peck, 1994; Hurst and Peck, 1996; Barton and Charlesworth, 1998; Peters and Lively, 2000). Most theories propose that sex is advantageous because it promotes linkage equilibrium between alleles at two or more loci (Felsenstein, 1988). An example of linkage disequilibrium would be if only genotypes *AB* and *ab* existed at two loci in a population, and *Ab* and *aB* are absent (or rarer than chance expectation). If *Ab* and *aB* are favored by selection, sex is advantageous because it can generate *Ab* and *aB* from *AB* and *ab*. Other models exist, but because the total number is large, they will not be reviewed here. Rather, I will focus on two well-known models for the advantage of sex, and describe their relevance for elucidating the origin of sex in segmented RNA viruses.

One classic model concerns the ability for sex to combat the harmful effects of deleterious mutations in small populations. Muller (1964) noted that in an asexual population there is a tendency for slightly deleterious mutations to accumulate. To illustrate this process, consider a population composed of classes containing 0, 1, 2 ... *k* deleterious mutations. If the mutation rate is high, mutation-free individuals become rare and they can be lost by genetic drift in small populations. In an asexual population the loss is irreversible, and the load of deleterious mutations increases with the successive loss of the least-mutated individuals. That is, the only way for an asexual population to reconstitute these lost classes is through back or compensatory mutations, which are expected to be especially rare in small populations.
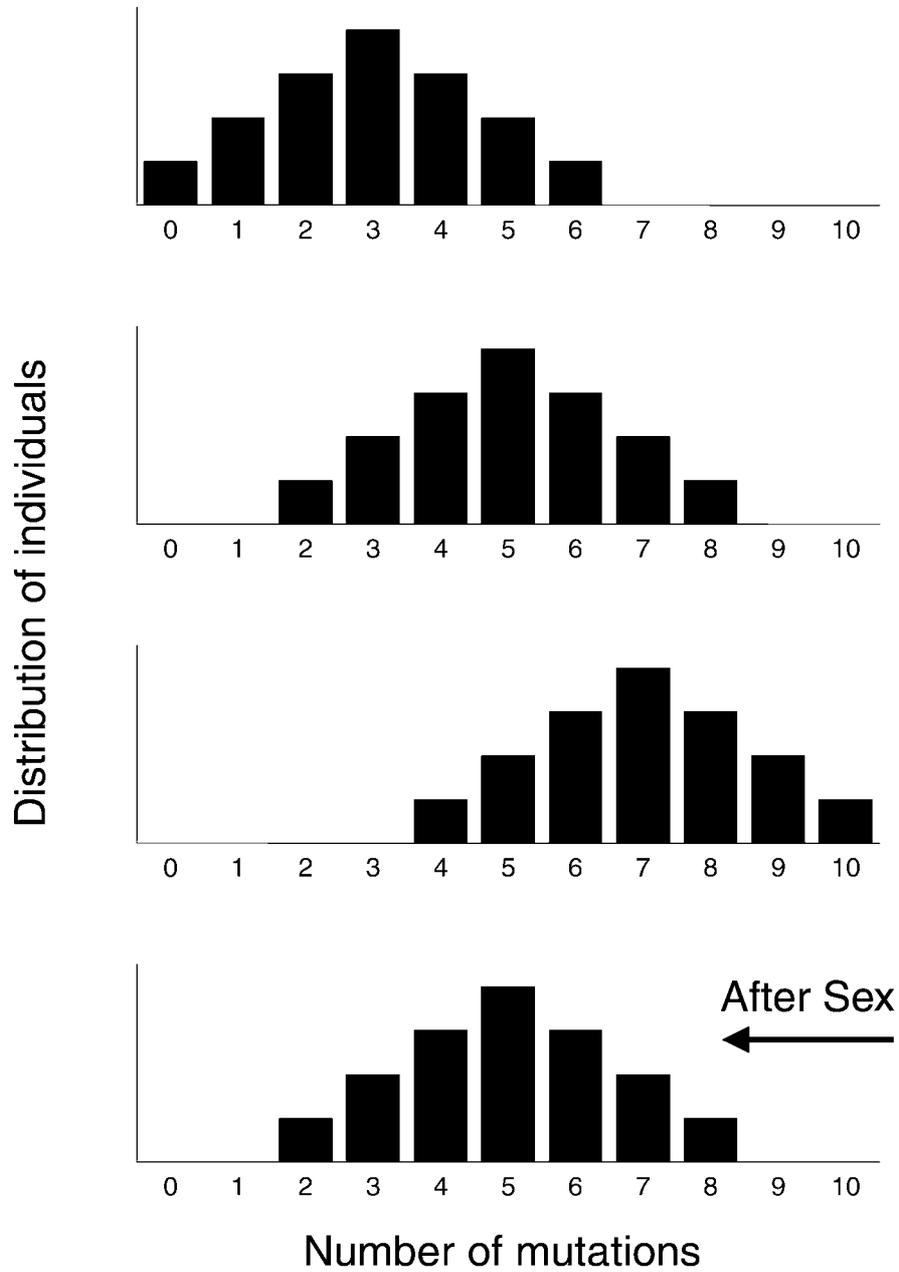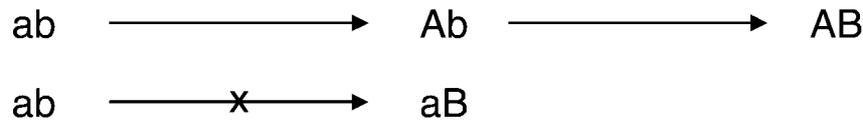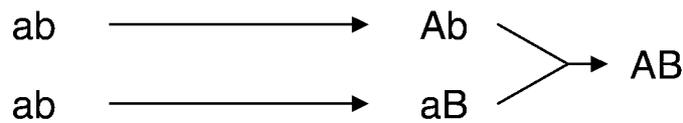
Figure 2. Muller's ratchet leads to loss of the least-mutated classes of individuals in an asexual population, but sex can reverse the process.

ab $\longrightarrow$ Ab $\longrightarrow$ AB

ab $\longrightarrow\!\!\times\!\!\longrightarrow$ aB

asexual population – sequential substitution

ab $\longrightarrow$ Ab

ab $\longrightarrow$ aB $\qquad$ AB

sexual population – genetic exchange

time $\longrightarrow$

Figure 3. In an asexual population the most favored genotype (*AB*) can arise only through sequential substitution in a single lineage, whereas genetic exchange allows sexual populations to combine mutations from separate lineages and speed the rate of adaptation.

Hence, this phenomenon has been termed 'Muller's ratchet' because it is similar to a ratchet tool that only clicks forward and cannot move in reverse. In contrast, sex can stop (or slow) Muller's ratchet because it allows two parents to exchange their genetic material and produce offspring that contain fewer harmful mutations (Figure 2).

The importance of Muller's ratchet is confined to small populations because the random loss of the best genotype due to drift becomes improbable in a large population. In contrast, the Fisher-Muller Hypothesis is a classic model that describes an advantage of sex in large populations (Fisher, 1930; Muller, 1932). This model argues that adaptation is more rapid in a large finite population of sexual organisms than in an equally large, but asexual, population evolving in the same environment (Figure 3). Once again, sex is beneficial because it promotes linkage equilibrium. Let the most favored combination of alleles in the population be *AB*. In an asexual population composed entirely of *ab* individuals, a favorable mutation can appear at either locus but these mutants will compete with each other until one or the other spreads to fixation (Gerrish and Lenski, 1998). Thus, adaptation is expected to occur through sequential substitution of mutations in a single lineage. In contrast, a sexual population features genetic exchange where the two mutations can occur in separate lineages but be combined in a single descendant. For this reason, the

Fisher-Muller hypothesis predicts that sex has the potential to accelerate the pace of adaptive evolution.

4. Testing the Advantage of Sex in Virus Populations

Many medically- and agriculturally-important pathogens are viruses that contain RNA as their genetic material; examples include HIV, poliovirus, and tobacco mosaic virus. RNA differs from DNA because RNA molecules are not proofread to correct the mistakes that occur during gene replication. For this reason, RNA viruses feature extremely high rates of genetic mutation (Drake and Holland, 1999), and most of these mutations are expected to be deleterious. Thus, Muller's ratchet should easily operate in small populations of RNA viruses, and sex may have evolved to facilitate the removal of harmful mutations from viral genomes. In addition, RNA viruses continually induce evolutionary responses in their hosts (or host immune systems), and are thus often exposed to novel or changing environments. Therefore, the Fisher-Muller Hypothesis may be relevant in RNA viruses because sex may have evolved to promote genetic variation that is useful when adaptation occurs in variable environments. Because these two models are not mutually-exclusive, it is possible to find evidence for the importance of each hypothesis in viral populations.

Although its natural host is unknown, the RNA bacteriophage (phage) $\phi6$ is a virus that can be grown in the laboratory on the bacterium *Pseudomonas phaseolicola*. Phage $\phi6$ has been sequenced and its life cycle has been the subject of considerable investigation (Mindich, 1988; Butcher *et al*., 1997). In addition, phage $\phi6$ is shown to be an extremely useful model for experimental evolution in RNA viruses (Chao, 1990; Turner and Chao, 1998; Burch and Chao, 1999). A $\phi6$ particle contains roughly 13 Kb (Mindich, 1988) of double-stranded RNA divided into three segments: S (small), M (medium), and L (large) (Semancik *et al*., 1973). Phage $\phi6$ features a three-stage life cycle that is typical of a lytic virus; infection, followed by replication of genetic material, and finally burst (lysis) resulting in death of the host cell and liberation of complete virus particles.

4.1. SEX OPPOSES THE ADVANCE OF MULLER'S RATCHET IN VIRUSES

To test whether Muller's ratchet can operate in phage $\phi6$, Chao (1990) passaged the virus in the laboratory using extreme bottlenecks consisting of only a single virus particle. This propagation scheme led to intensified genetic drift in the small virus populations, producing genotypes bearing many deleterious mutations. After 40 passages involving bottlenecks, the fitness (in terms of growth rate) of an evolved virus relative to its un-evolved ancestor was measured; fitness above or below 1.0 indicates that the virus is, respectively, greater or lesser fit than its ancestor. Results showed that the mutated viruses featured fitness values much less than 1.0,
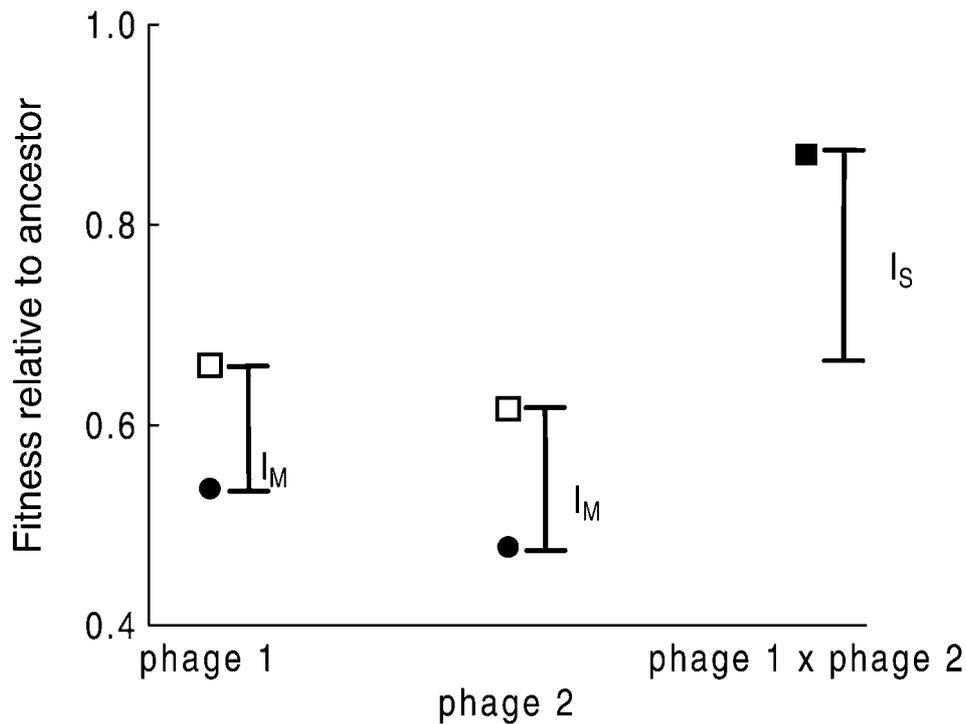
Figure 4. A hybrid cross between two phages of low fitness demonstrates that sex can combat the mutational load; I_M: improvement due to mutation in a selfed-cross, I_S: improvement due to sex in a hybrid cross; after Chao *et al.* (1997).

indicating that deleterious mutations had appeared, and severely limited ability of the viruses to grow on the host bacterium. In addition, these results showed that deleterious mutations are clearly more common than both back and compensatory mutations.

To determine whether segment reassortment combats Muller's ratchet, Chao *et al.* (1997) crossed (mated) the mutated viruses with one another. Sex is advantageous if the cross yields a hybrid population that contains a hybrid (reassortant) whose fitness is greater than either of the two parents. Because locating such a hybrid in a cross is difficult (i.e., in this experiment reassortants were produced at a low frequency of 5–20%), the authors enriched for such genotypes by subjecting the hybrid population to mass selection. This was achieved by passaging the population through several growth cycles featuring a larger bottleneck of $\sim 10^3$ phages. With a larger bottleneck selection operates with minimal drift, and hybrid populations containing higher fitness reassortants should evolve a higher fitness, yielding a measure of the improvement due to sex (I_S). To control for the possibility that a hybrid population improves because of back and compensatory mutations, the parent strains were 'self-crossed' and subjected to mass selection, yielding

a measure of the improvement due to mutation (I_M). Figure 4 shows a typical result from these experiments where sex led to the formation of hybrid genotypes featuring a greater fitness than that of either parent. Improvement ($I_S > 0$) could only have occurred if deleterious mutations had appeared on separate segments; that is, if harmful mutations were confined to a single segment (a hotspot), then segment reassortment would provide no advantage. Because these data confirm that reassortment can slow or reverse the debilitating fitness effects of Muller's ratchet, it suggests that reassortment evolved as a mechanism to combat the buildup of harmful mutations. However, Muller's ratchet is only expected to operate in small populations, emphasizing a need to examine whether reassortment is also beneficial in virus populations of large size.

4.2. SEX CAN SLOW ADAPTATION IN LARGE VIRUS POPULATIONS

To test the Fisher-Muller Hypothesis in RNA viruses, Turner and Chao (1998) predicted that in a novel environment sexual populations of $\phi 6$ should evolve more rapidly than their asexual counterparts. Mode of reproduction was controlled by manipulating the multiplicity of infection (m.o.i.), or ratio of infecting phages to host cells. Assuming Poisson sampling (Sokal and Rohlf, 1995) 97% of all infected cells contain two or more viruses at m.o.i. = 5, and reproduction is primarily sexual. In contrast, only 0.1% of all infected cells contain two or more viruses at m.o.i. = 0.002, and reproduction is primarily asexual. A single clone of wild type $\phi 6$ was divided into three high multiplicity (m.o.i. = 5) and three low multiplicity (m.o.i. = 0.002) populations, and allowed to evolve on *P. phaseolicola*. After 250 generations (50 days) of viral evolution, population samples (stored in the freezer) were competed against the common ancestor to measure changes in fitness.

Fitness trajectories of sexually- and asexually-evolved populations were obtained by plotting the grand mean fitness of each treatment group over time (in 50 generation increments). Figure 5A shows that the viruses increased in fitness relative to their common ancestor, indicating that beneficial mutations led to fitness improvements in the populations. However, contrary to predictions, sex did not augment the rate of fitness improvement. Asexual populations featured a positive linear increase in fitness over time, and achieved the highest endpoint fitness values. In contrast, the fitness trajectory of sexual populations was concave; these populations appeared to quickly reach a selective plateau that was followed by a fitness decline. These data suggest that the imposed level of sex was costly in phage $\phi 6$. But the odd fitness trajectory observed in the sexual populations hinted that a more complex explanation existed for deviation from the Fisher-Muller Hypothesis.

The standard assay (Chao, 1990) used to measure fitness in Figure 5A compares virus growth rates during strictly clonal infections, where viruses do not interact within the cell. But intracellular interactions are necessary for virus sex (Figure 1); thus the viruses evolved at high multiplicity experienced an environment radically different from that of the standard assay. To examine the importance of this en-

(A) *CLONAL INFECTIONS*
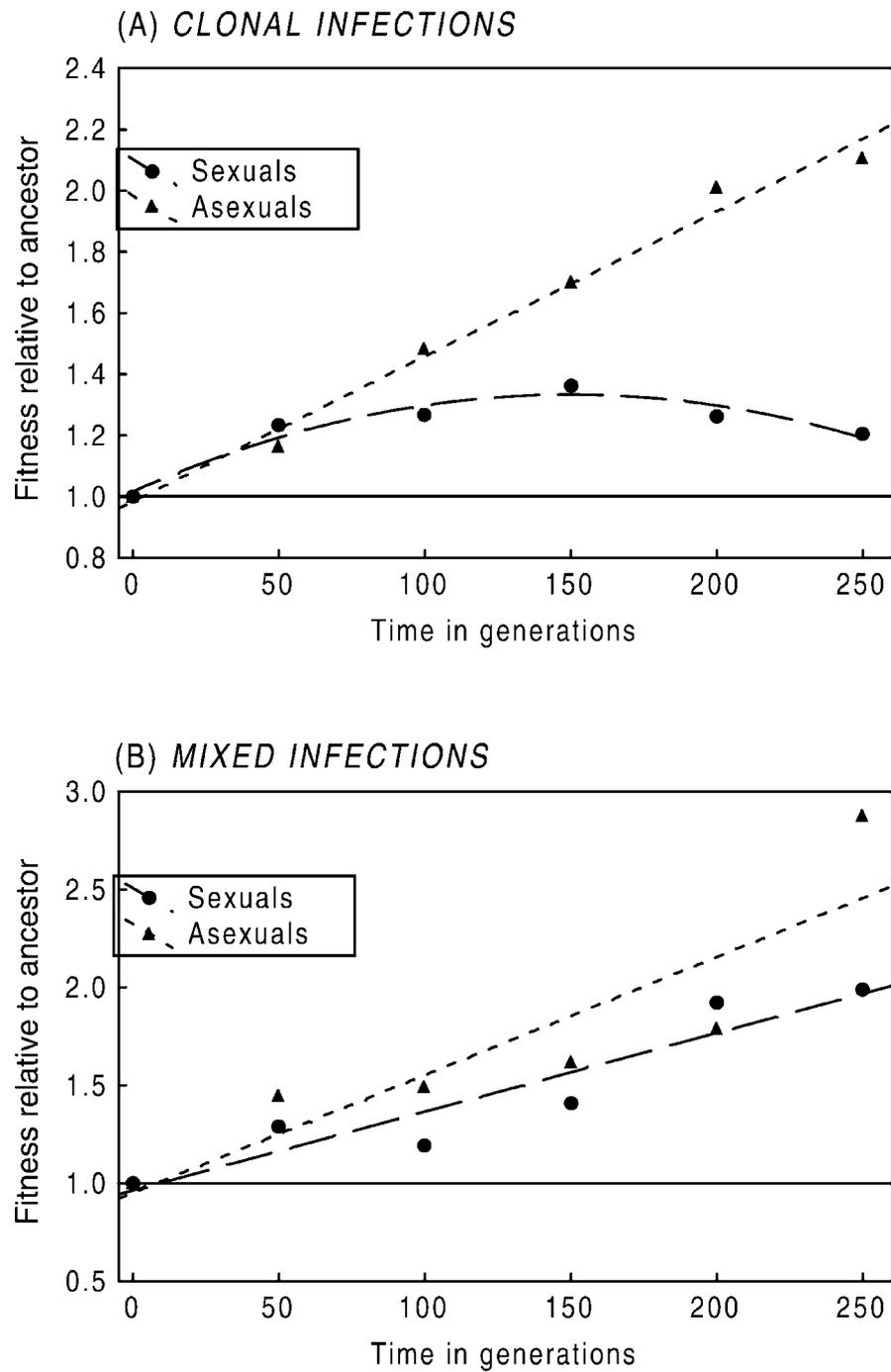
(B) *MIXED INFECTIONS*

Figure 5. A: Contrary to the Fisher-Muller Hypothesis, sex does not increase the rate of fitness improvement in virus populations. B: Sexually-evolved viruses are strong competitors in their evolved environment (mixed infections), but their rate of fitness improvement still lags behind their asexually-evolved counterparts; after Turner and Chao (1998).

vironmental difference, Turner and Chao (1998) measured fitness of the evolved populations at m.o.i. = 5. Figure 5B shows that the sexually-evolved viruses perform much better when competing during mixed infections. This indicates the viruses evolved traits specific for co-infection, but detrimental for clonal infection (see also Sevilla *et al.*, 1998). The tradeoff associated with these traits is very obvious by late in the experiment when a downturn occurs in the fitness trajectory of sexually-evolved viruses during clonal infections. More importantly, Figure 5B reveals that endpoint fitness still ranks higher in the asexually-evolved viruses, in continued violation of the Fisher-Muller prediction.

These surprising results can be explained by the evolution of selfishness when viruses are propagated at high multiplicities. During infection viruses manufacture products that diffuse within the cell, preventing an individual virus from exclusive access to its own gene products. This creates a conflict of interest whenever multiple viruses infect a single host (Nee and Maynard Smith 1990). A virus that synthesizes less but specializes in appropriating a larger share of the products can be defined as selfish (Turner and Chao, 1999). Because the phages cultured at high multiplicities gain an added advantage *only* in mixed infections, this suggests that these viruses evolved a selfish strategy for intracellular competition. The drawback is that selfish genes (or closely linked non-selfish genes at other loci) are inferior when the viruses compete in alternate environments where intrahost interactions are less important. The lesson from these experiments is that too much sex can be a bad thing for phage $\phi6$; abundant sex allows promiscuous viruses to selfishly ensure that their inferior genes reach subsequent generations (Turner and Chao, 1999).

5. Conclusions and Future Work

The above experiments provide strong evidence that sex may have originated in RNA viruses because genetic exchange is useful in combating mutational load. Of course, this is contingent upon population size in phage $\phi6$ being small enough that genetic drift becomes important; unfortunately, no information exists yet for effective population sizes of $\phi6$ in nature. In contrast, the above studies provide insufficient evidence for evaluating whether the Fisher-Muller Hypothesis is important in the virus. Whereas the high levels of sex imposed on viral populations led to evolution of traits that were not generally beneficial, it is unknown whether moderate (or even low) levels of sex are sufficient to provide an advantage in large populations evolving in novel environments. Further experiments are needed to confirm this possibility, and to examine the relevance of more recent models for the advantage of sex; e.g., whether sex originated in viruses because it provides a large advantage when mutations tend to interact synergistically through epistasis (Kondrashov, 1993). Although phage $\phi6$ provides an excellent model for studying the evolution of sex in RNA viruses, relatively few experiments have examined

sexual mechanisms in DNA viruses (e.g., Malmberg, 1977) and there is clearly a pressing need for further work. In addition, the frequency of recombination, and the number of genome segments per virus particle can be manipulated in laboratory systems (e.g., Onodera *et al*. 1998), allowing experimental tests of the effects of these variables.

There is a potential added cost of sex in segmented RNA viruses that has not been fully evaluated. The two-fold cost of sex (see above) applies to species composed of males and females, where (all else being equal) each of the two parents contributes an equal share of genetic material to the sexually-produced offspring. Thus, an even greater cost of sex should be expected in systems containing three or more parents; this has been used to argue why the overwhelming majority of sexually-reproducing species are bi-parental (Fisher, 1930). An analogous situation occurs when multiple RNA viruses co-infect a single host cell, producing large numbers of sexual partners during reassortment. One might expect that a large cost of sex ensues because an individual virus stands to contribute very little genetic information to the next generation. We have previously argued that limits to co-infection in viruses may have evolved to reduce the strength of competition for intracellular resources (Turner *et al*., 1999). Similarly, the observed limits could have originated because a multi-fold cost of sex produces strong selection for reduced numbers of viruses co-infecting the cell. For instance, phage $\phi6$ features an exclusion mechanism where two to three viruses (on average) infect the cell, even though up to 50 viruses can attach to the cell (Olkkonen and Bamford 1989). This trait may have evolved because it allows entry by more than one virus to achieve sexual reproduction, but prevents very many partners from taking part in segment reassortment. There is a need to examine further hypotheses concerning this intriguing cost of sex in segmented viruses from a theoretical standpoint, and for empirical data that test the predictions.

Recombination may have evolved as a mechanism that promotes genetic exchange. But it is arguable that recombination originally evolved to facilitate repair of damaged genetic material, and that sex is simply the by-product of this process (Maynard Smith, 1978). Similarly, it has been suggested that reassortment did not originally evolve to promote sex. Rather, segmentation of the RNA genome may have evolved as a means to facilitate packaging of viral RNA into protein capsids during intracellular replication, and reassortment occurs as a side-effect (Qiao *et al*., 2000). Because the origin of segmentation in RNA viruses is contentious, further studies are needed to resolve this question. It is important to note that however segmentation arose in the past, this does not mean that segmentation has no value as a sexual mechanism in the present. This is because evolution frequently modifies extant features to serve new functions.

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