ORIGINAL ARTICLE

doi:10.1111/j.1558-5646.2007.00205.x

HOST DENSITY IMPACTS RELATIVE FITNESS OF BACTERIOPHAGE Φ6 GENOTYPES IN STRUCTURED HABITATS

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Received August 14, 2006

Accepted June 25, 2007

Spatially structured environments may impact evolution by restricting population sizes, limiting opportunities for genetic mixis, or weakening selection against deleterious genotypes. When habitat structure impedes dispersal, low-productivity (less virulent) infectious parasites may benefit from their prudent exploitation of local hosts. Here we explored the combined ability for habitat structure and host density to dictate the relative reproductive success of differentially productive parasites. To do so, we allowed two RNA bacteriophage Φ6 genotypes to compete in structured and unstructured (semi-solid versus liquid) habitats while manipulating the density of *Pseudomonas* hosts. In the unstructured habitats, the more-productive phage strain experienced a relatively constant fitness advantage regardless of starting host density. By contrast, in structured habitats, restricted phage dispersal may have magnified the importance of local productivity, thus allowing the relative fitness of the less-productive virus to improve as host density increased. Further data suggested that latent period (duration of cellular infection) and especially burst size (viral progeny produced per cell) were the phage "life-history" traits most responsible for our results. We discuss the relevance of our findings for selection occurring in natural phage populations and for the general evolutionary epidemiology of infectious parasites.

KEY WORDS: **bacteria, competition, habitat structure, phage, *Pseudomonas*, selection, fitness, virus.**

Competition intensity, mate access, parasite avoidance, and prey escape can all depend on the ability of organisms to move, actively or passively, through the environment. Habitats that tend to limit the dispersal of individuals or their offspring can impact their reproductive success and, by extension, the course of organismal evolution (e.g., Bell et al. 1991; Tilman 1994; Epperson and Li 1997; Petren and Case 1998). For example, insect community diversity may hinge on spatial separation of resources; independent aggregation of insect species facilitates their coexistence because this allows competition within species to exceed that occurring among species (see review by Hanski 1990).

Habitat spatial structure is particularly important for microbial populations because individuals from a single species can experience widely varying habitats, such as bacteria forming dense aggregations on solid surfaces versus thriving in a water column. Indeed, previous studies suggest that habitat structure can promote microbial diversity (e.g., Korona et al. 1994; Rainey and Travisano 1998; Habets et al. 2006; Kerr et al. 2006). Chao and Levin (1981) examined the influence of habitat structure on competition between an *Escherichia coli* strain that produced a colicin, and an *E. coli* strain that was sensitive to this antibacterial toxin. In unstructured (well-mixed liquid) habitats, colicinogenic *E. coli* were able to displace sensitive *E. coli*, but only if the colicin producers (and hence their toxins) were sufficiently common at the outset. Otherwise, the colicin producers were driven extinct due to their

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intrinsic growth disadvantage relative to the sensitive bacteria. By contrast, structured (i.e., solid agar) environments allowed colicin producers to more easily invade when rare; locally dense assemblages (colonies) of producers released sufficient toxin to eliminate neighboring sensitive bacteria.

The growth of bacteriophages is also impacted by habitat structure. In well-mixed liquids, lytic (lethal) phages randomly collide with bacteria, allowing cell attachment and entry, intracellular replication and transcription of phage genetic material (RNA or DNA), and release of ~100 or more phage progeny from the cell. Therefore, in an unstructured environment, such as a shaking culture flask, phages may encounter and kill all available sensitive hosts. Phage growth under increased habitat structure differs because more-viscous environments can limit phage diffusion and immobilize bacteria. For example, in semi-solid agar, phage progeny exit the cell and infect the neighboring bacteria, causing a localized viral plaque (hole) to form on the bacterial lawn growing in the three-dimensional agar matrix.

The current study is novel in exploring the interplay between habitat structure and host density on the relative growth of phage genotypes, and relates to current theoretical work on selection for parasite strategies according to habitat structure. Recent models predict that habitat structure can benefit parasites of low productivity, where productivity is defined as the number of parasite progeny produced per infection (Claessen and De Roos 1995; Boots and Sasaki 1999; van Baalen 2002; Boots et al. 2004); however, relatively few experiments have addressed the issue (e.g., Kerr et al. 2006; Boots and Mealor 2007). If hosts and parasites freely mingle, parasites are unlikely to be surrounded by hosts they have already infected, and the most productive parasite should spread fastest both locally and globally. By contrast, when habitat structure restricts movement, infected hosts are soon surrounded by other infected hosts, which may permit more prudent (e.g., less productive) parasites to be locally successful.

Although we did not test these predictions explicitly, we used two phage genotypes that differed markedly in reproductive ability to test whether the less-productive phage fared better in structured than in unstructured habitats when host density was manipulated. For simplicity, we confined our experiments to bacterial infections occurring under conditions of low multiplicity (ratio of viruses to host cells). Here, different phage genotypes tend not to infect the same host cell. (In both structured and unstructured habitats, conditions may change through time such that viruses outnumber host bacteria, increasing the likelihood of coinfection and causing within-host interactions among different genotypes to become important [Turner and Chao 1998, 1999; Froissart et al. 2004]. Our experiments specifically limited this possibility.) Recent studies have examined phage evolution in structured or unstructured habitats (Abedon et al. 2003; Lythgoe and Chao 2003; Forde et al. 2004; Harcombe and Bull 2005; Brockhurst et al. 2006), but, to our knowledge, no study has simultaneously examined effects of habitat structure and host density on relative performance (fitness) of phage genotypes.

Using infection of *Pseudomonas syringae* pathovar *phaseolicola* by the RNA phage Φ6 (family *Cystoviridae*; Vidaver et al. 1973; Mindich 1988), an increasingly popular laboratory model for examining questions in ecology and evolution (Froissart et al. 2004; Montville et al. 2005; Duffy et al. 2006; Dennehy et al. 2007; Turner and Duffy 2008), we estimated the fitness of a less-productive Φ6 strain relative to wild-type Φ6 in the presence/absence of habitat structure while manipulating initial host density. Our results supported the idea that habitat structure can increase the fitness of less-productive phages relative to more-productive phages, perhaps owing to effects of local host density on phage growth and competition in these environments. Estimates of phage growth characteristics ("life-history traits") suggested that greater fecundity was the prime contributor to the fitness advantage of the wild-type virus.

Methods

STRAINS

Pseudomonas syringae pathovar *phaseolicola* (hereafter PP) is a bacterial pathogen that infects wild and cultivated legumes. Our strain of PP was purchased from American Type Culture Collection (# 21781), and is a nonmotile bacterial genotype. LM1034 is PP containing plasmid pLM746, which encodes the omega fragment of the *E. coli* β-*galactosidase* gene (Onodera et al. 1993; see also Froissart et al. 2004). PP is the typical laboratory host for phage Φ6, a lytic virus with three dsRNA segments (large, medium, and small) per particle (Vidaver et al. 1973; Mindich 1988). Our strain of Φ6 is a single clone derived from the stock of L. Chao (University of California, San Diego). $\Phi 6_M$ is a mutant created by inserting the alpha fragment of the *E. coli* β-*galactosidase* gene into the large segment, and consequently, its fitness is reduced relative to wild-type Φ6 (Froissart et al. 2004). On agar inoculated with 0.4% X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) and 200 μl (8×10^8 cells) of overnight LM1034 culture, $\Phi 6_M$ and Φ6 form blue and colorless plaques, respectively. The X-Gal marker for $\Phi 6_M$ is extremely stable (Froissart et al. 2004), such that genetic revertants (β-*galactosidase* gene deletions or inactivations) could be ignored in our experiments.

CULTURE CONDITIONS

All phages and bacteria were cultured at 25°C in LC medium (i.e., Luria broth; 10 g NaCl, 10 g Bacto® tryptone, and 5 g Bacto® yeast extract per liter at pH 7.5). Bacterial cultures were grown from a single colony placed in a 50-mL flask containing 10-mL LC medium and incubated with shaking (120 rpm). After 24 h,

bacterial cultures attained stationary phase densities ($\sim 4 \times 10^9$ cells mL^{-1}). All bacterial stocks were stored at $-80^\circ C$ in a 2:3 glycerol/LC solution.

Viruses were cultured by placing phage and stationary phase bacteria into 3 mL of 0.7% top agar (liquid at 45°C, solid at 25°C); the resulting solution was then overlaid onto a 1.5% agar plate. *Pseudomonas* bacteria are nonmotile in 0.7% agar overlays regardless of strain motility. After 24 h incubation at 25°C, viruses formed visible plaques in the bacterial lawn growing in the overlay. Cell-free lysates of virus were prepared by removing the top agar, resuspending it in 3 mL LC broth, and centrifuging it at 3000 rpm for 10 min. The supernatant was then filtered (0.22 μm, Durapore®; Millipore, Bedford, MA) to remove bacteria. Lysates were stored at −20°C in a 4:6 glycerol/LC (v/v) solution.

PHAGE GROWTH

Plaque size assays

In each assay block ($N = 3$), ~100 particles from freshly prepared lysates from either Φ6 or $\Phi 6_M$ were mixed with $\sim 2 \times 10^8$, 8×10^8, 2×10^9 or 4×10^9 PP cells in 3 mL top agar. Agar plates were preweighed and standardized to avoid confounding effects of agar thickness on plaque size (C. Burch, pers. comm.). Plaques were digitally photographed, and bitmap images were analyzed using Image J, version 1.33u for Windows (http://rsb.info.nih.gov/ij/). A macro was used to calculate the number and area (mm^2) of plaques on experimental plates. An analysis of covariance (ANCOVA) was performed to examine effects on plaque size (the dependent variable) of phage genotype, $\log_{10}$ initial host density, and their interaction.

Titer assays in liquid

Approximately 200 particles of Φ6 or $\Phi 6_M$ were added to a 50-mL flask containing 6 mL LC and 4×10^8, 8×10^8, 1.6×10^9, 2.4×10^9, or 8×10^9 PP cells, with threefold experimental replication; the mixtures were then incubated with shaking (120 rpm). Note that the volumes and population sizes were twice those in the plaque size assays to ensure efficient mixing of microbes in liquid culture, but the range of phage-to-host ratios was identical. After 24 h, mixtures were filtered to remove bacteria and the supernatant was titered on PP to estimate total phage growth. An ANCOVA was performed to examine effects of genotype, $\log_{10}$ initial host density, and their interaction on the dependent variable, final $\log_{10}$ titer.

BACTERIAL LAWN GROWTH

Volumes containing 2×10^8, 8×10^8, or 2×10^9 overnight PP cells were mixed with 3 mL top agar with four-fold replication. At 0, 3, 4, 5, 6, and 24 h, a 13-mm diameter plug of top agar was removed from each replicate and resuspended in 3 mL LC broth with gentle vortexing. Serial dilutions were plated on LC agar, and colonies visible at 48 h were counted to estimate cell densities. Log_{10} densities between 3 and 6 h were regressed against time to calculate the rate of bacterial growth (i.e., slope of the regression), and an ANCOVA was performed to examine the effects of $\log_{10}$ initial host density on the dependent variable, slope. Mean bacterial carrying capacity (the maximum population size allowed by the resources in the habitat) in each treatment was estimated using $\log_{10}$ densities at 24 h.

PHAGE FITNESS

Fitness in structured habitats

We used paired-growth assays (Chao 1990) to measure fitness of $\Phi 6_M$ relative to Φ6 at different initial densities of host bacteria in structured habitats. The two genotypes were mixed 1:1 and samples containing ~250 total viruses were combined with $\sim 1 \times 10^8$, 2×10^8, 3×10^8, 4×10^8, 5×10^8, 6×10^8, 8×10^8, 1×10^9, 2×10^9, or 4×10^9 PP cells and 3 mL top agar. The initial phage-to-host ratios therefore spanned 2.5×10^{-6} and 6.25×10^{-8}. Each host density treatment was replicated five- to ten-fold. A preliminary experiment used inocula of only three host densities (2×10^8, 8×10^8, and 2×10^9) replicated fivefold, whereas a subsequent experiment contained all 10 inocula, also replicated fivefold. In addition, ~150 total viruses from each mixture were added to 3 mL top agar with 200 μl LM1034 cells and grown on an X-gal plate. The resultant blue and clear plaques were counted to confirm a 1:1 starting ratio (R_0) of viruses at time zero. After 24 h, plaques from each experimental plate were collected to obtain a lysate, which was titered on X-Gal agar to estimate the genotypic ratio in the harvested lysate (R_1). Thus, relative fitness was determined on PP lawns, but starting and final ratios were assayed on LM1034. Consistent with previous studies using Φ6 (e.g., Chao 1990; Turner and Chao 1998, 1999; Montville et al. 2005), fitness (W) is defined as

$$W = R_1/R_0.$$

We then $\log_{10}$-transformed fitness values for normality and presentation below. If $\log_{10} W \neq 0$, then the two genotypes differ in relative fitness.

Fitness in liquid

To estimate fitness in an unstructured habitat, we mixed the phage genotypes as described above, with threefold experimental replication. A sample of each mixture containing ~500 total viruses was then combined in a sterile flask with 6 mL LC and $\sim 2 \times 10^8$, 4×10^8, 6×10^8, 1×10^9, or 4×10^9 PP cells, such that the initial phage-to-host ratios were identical to a subset of those employed in the structured habitat assays. The larger volumes ensured efficient mixing of liquid cultures when incubated with shaking (120 rpm). After 24 h, cell-free lysates were prepared and fitness was assayed using X-gal plates as above.

BURST ASSAYS

Viral growth parameters were measured using single-burst experiments (Stent 1963; Chao et al. 2002; Dennehy and Turner 2004). Approximately 4×10^5 phages were mixed with 4×10^8 exponentially growing PP cells in 1 mL LC to yield initial phage-to-host ratios of 0.001. Viruses were then allowed to undergo a single infectious growth cycle (180 min) with incubated shaking; at 40 min (sufficient time for virus adsorption) the mixtures were diluted, ensuring that bacteria did not reach stationary phase during the assays (Chao et al. 2002). Throughout the infection process, samples were plated on PP lawns to estimate phage titer (N_i, plaque forming units per milliliter). Samples were obtained a minimum of ten times during the growth cycle, for example, $i = 0$, 40, 50, 70, 90, 100, 110, 160, 170, and 180 min. Maximal growth rate (m) was the slope obtained by regressing the natural logarithm (ln) of N_i against time during the period over which phages were released from infected bacteria (the rise period). We also computed mean titer following bacterial lysis (N_S). Assuming that the vast majority of input viruses infect a single cell at a phage-to-host ratio of 0.001 (Turner et al. 1999), burst size (B) is the average number of progeny produced per infected cell: $B = (\text{mean } N_S)/(4 \times 10^5)$. The latent period ($L$) is defined as the time at which virus progeny are released into the environment, and was calculated as the intercept of m and the regression line calculated using $\ln N_i$ values during the prerise period. Burst assays for the genotypes were conducted in parallel to eliminate any confounding effects of host cell culture. t-tests were used to determine significant differences in growth parameters between Φ6 and $\Phi6_M$.

ATTACHMENT ASSAYS

To measure the rate of phage attachment to hosts in liquid (Stent 1963) as host density varied, 4×10^3 virus particles were mixed with 5×10^7, 2×10^8, or 5×10^8 exponentially growing PP cells in 10 mL LC medium with threefold replication. The mixture was incubated with shaking for 40 min. Immediately after mixing and every 10 min thereafter, a 500-μl sample from the mixture was centrifuged at 5000 rpm for 1 min to pellet the cells and the supernatant was titered on PP lawns. Because attached phages were pelleted with the bacterial cells, only unattached phages remained in the supernatant. At each time point we calculated R_i, the ratio of plaques at time $t = i$ relative to the initial number of plaques. We then obtained the slope of the regression line for $\ln R_t$ versus time. The slope is equal to $-kC$, where k is the attachment rate constant and C is the concentration of host cells in the mixture. Attachment rate (k) units are per milliliter per cell (or per phage) per minute. A two-way ANOVA was performed with attachment rate as the dependent variable and genotype and host density as fixed factors.

Results

STRUCTURED HABITATS

Effect of host density on plaque size

Initial host density should impact phage plaque size in three ways. First, variance in the timing of plaque initiation, and therefore in final plaque size, should increase as host densities decline (unless phage preadsorption is employed; Abedon and Yin 2008). Second, rates of plaque size increase may vary as a function of initial host density, with increasing initial host densities leading to faster, then slower, plaque growth rates (Mayr-Harting 1958; Burch and Chao 2004). Third, the interval between plaque initiation and lawn entrance into stationary phase will be shorter the higher initial bacterial densities (Kaplan et al. 1981), resulting in smaller plaques if initial lawn bacterial densities are high. Although these factors should qualitatively affect all phage genotypes similarly, their effects among different phage genotypes could vary quantitatively.

To test whether increased densities of host bacteria could differentially affect the plaque size associated with our two phage genotypes, we conducted blocked ($N = 3$) experiments measuring 24 h plaque sizes of Φ6 and $\Phi6_M$ at four initial host densities ranging between 2.0×10^8 and 4.0×10^9 cells per plate (note that these densities fall within the range expected to yield an inverse relationship between plaque size and initial host density; Burch and Chao 2004). Approximately 100 viral particles were seeded per plate to reduce the likelihood of plaque overlap, hence minimizing the probability of coinfection. A total of 588 Φ6 plaques and 624 $\Phi6_M$ plaques were independently analyzed. We detected a significant block effect on plaque size (assay day; $F = 64.59$, $\text{df} = 2$, $P < 0.0001$), which is not surprising given the sensitivity of plaque size to uncontrollable factors such as ambient humidity and physiological state of the host lawn (C. Burch, pers. comm.). However, this result did not impact the patterns observed in the data, allowing us to remove the block from the analysis. Generally speaking, mean plaque size declined for Φ6 and for $\Phi6_M$ as initial host density increased (Fig. 1). We observed statistically significant effects on plaque size of genotype (ANCOVA; $F = 9.33$, $P = 0.0023$), $\log_{10}$ host density ($F = 104.91$, $P < 0.0001$), and their interaction ($F = 7.72$, $P = 0.0055$). The significant interaction term indicated that the viruses differed in their diminishing plaque size as host density increased; Φ6: slope $= -1.45$, $t = 9.14$, $P < 0.0001$; $\Phi6_M$: slope $= -0.83$, $t = 5.32$, $P < 0.0001$. We concluded that increasing host density negatively impacted plaque size of both viruses, but that the absolute change in plaque size differed between the genotypes across the treatments.

Effect of plaque size on particles per plaque

We sought to determine whether larger plaque sizes tended to produce greater numbers of viral particles within plaques. To do so, we seeded ~100 particles of Φ6 or $\Phi6_M$ onto host lawns that ranged in initial density between 2.5×10^7 and 1.0×10^9 host

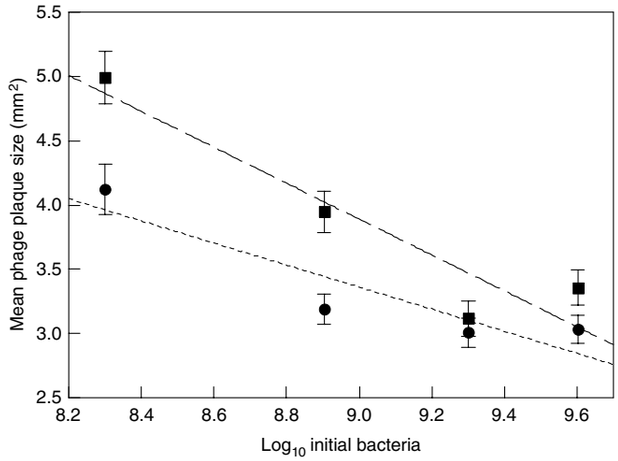

Figure 1. **Mean plaque sizes of two phage genotypes decline with increasing initial densities of host bacteria (*P. phaseolicola*) in structured habitats. Each point is the mean of between 119 and 173 independent measurements for phage Φ6 (filled squares, dashed line) or $\Phi6_M$ (filled circles, dotted line). Error bars are ±SE, and lines are least-square regressions. See text for statistical analyses.**

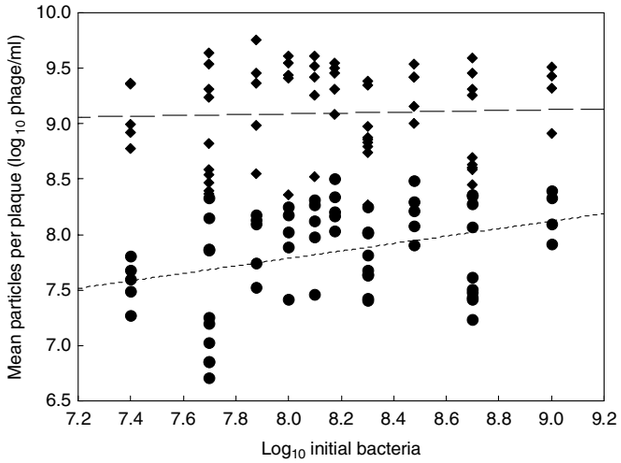

Figure 2. **Mean number of viral particles per plaque as a function of initial host density in structured habitats. Each point is the average number of viable particles for Φ6 (filled squares, dashed line) or $\Phi6_M$ (filled circles, dotted line) when viruses are grown independently. Φ6 produces roughly the same number of progeny per plaque across host densities regardless of plaque area, whereas $\Phi6_M$ tends to produce more progeny within plaques of small size when initial bacterial densities are high (cf. Fig. 1). Lines are least-square regressions. See text for statistical analyses.**

cells per plate. We counted the number of plaques formed after 24 h, harvested these plaques, and titered the resulting lysate to calculate the average number of phage particles per plaque (PPP, in units of mean plaque forming units per plaque). We regressed $\log_{10}$ PPP against $\log_{10}$ host density for each virus (Fig. 2: $\Phi6_M$: slope = 0.336; Φ6: slope = 0.037). A simultaneous analysis of the slopes rejected the null hypothesis that the slopes were equal ($t = 2.66$, $P = 0.0088$). This analysis suggested that Φ6 produced a constant number of particles regardless of the associated plaque area, whereas $\Phi6_M$ produced more PPP as initial host density increased. We concluded that the relatively faster epidemic spread of Φ6 through space did not always translate to a large advantage in the number of infectious particles produced in the time allowed (see further discussion below).

Bacterial lawn growth

Plaque size should be constrained by the time available for host growth in the structured habitat. As initial host density increases, the bacteria should reach stationary phase earlier in the incubation, limiting the time available for epidemic spread (plaque size area). The limitation arises because stationary phase bacteria often are less competent (physiologically supportive) for phage infection and/or replication (Rabinovitch et al. 2002; Abedon and Yin 2008). This process should straightforwardly impact our structured habitat experiments assuming that PP lawns of differing initial density achieve the same carrying capacity, and that they approach this value by exponentially growing at similar rates. To confirm these assumptions, we performed replicated ($N = 4$) assays in which lawns were seeded with three different cell densities (see Methods), and sampled through time to estimate changes in cell density. Results (Fig. 3) showed that the rates ($\Delta\log_{10}$ density h^{-1}) of bacterial growth were similar across treatments. To determine if bacteria grew identically in the treatments, we regressed $\log_{10}$ bacterial abundance versus time and tested whether the slopes of the regressions were equivalent. We found no statistically significant effect of $\log_{10}$ initial host density on the dependent variable, slope (ANCOVA; $F = 1.28$, $P = 0.28$).

In addition, we found that the grand mean of stationary phase density was 2.47×10^9 cells $mL^{-1} \pm 6.03 \times 10^8$ SE ($N = 12$), a value slightly lower than that achieved by the bacteria in liquid ($\sim 4 \times 10^9$ cells mL^{-1}). More importantly, the stationary phase density on plates did not differ according to initial bacteria density (ANCOVA; $F = 0.658$, $P = 0.54$). The Figure 3 inset shows the hypothetical approach to carrying capacity for the treatment populations of bacteria, based on extrapolations of the linear regressions for exponential phase data. We note that this analysis is inherently inaccurate because it naively assumes that the approach to stationary phase is strictly linear for the treatment populations. Rather, a more complex (e.g., curvilinear) function may better describe the approach to carrying capacity. However, the analysis is useful for highlighting the expected difference in time of entry to stationary phase among treatments. We concluded that high host densities contributed to the resultant smaller plaque sizes by causing reduced time for phage propagation.

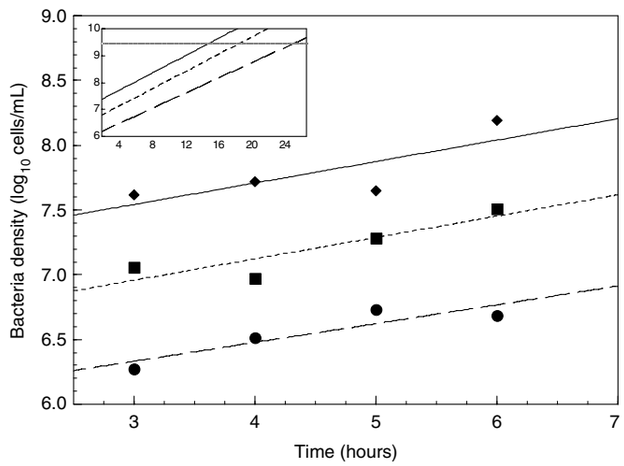

Figure 3. Growth of uninfected *P. phaseolicola* bacteria occurs similarly in the structured habitat at three different initial cell densities (filled circles: 6.67×10^7 cells mL^{-1}; filled squares: 2.67×10^8 cells mL^{-1}; filled diamonds: 1.33×10^9 cells mL^{-1}). Points are mean values ($N = 4$), and linear fits to the data are shown. The inset shows the hypothetical times at which cell growth reaches carrying capacity (i.e., enters stationary phase; dotted line), assuming constant exponential growth. See text for details.

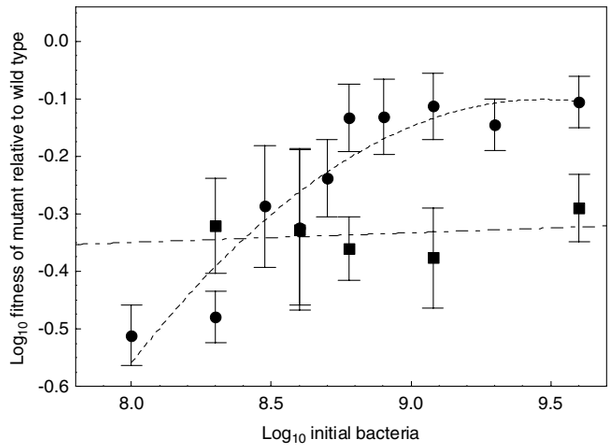

Figure 4. Log_{10} fitness of $\Phi6_M$ relative to $\Phi6$ is a saturating function of $\log_{10}$ initial host density in structured habitats, but is constant in unstructured habitats. Filled circles ($\pm$SE) represent the means ($5 < N < 14$) of independent fitness estimates in structured environments; dotted line is the fit to the data using a quadratic regression. Filled squares ($\pm$SE) represent the means ($5 < N < 14$) of assays in unstructured environments; dashed line is the least squares regression. See text for details and statistical analyses.

Phage fitness at different host densities

In structured habitats, $\Phi6_M$ was less disadvantaged in plaque size and PPP when initial host densities were high (Figs. 1, 2). These fecundity differences should translate to improved fitness of $\Phi6_M$ relative to $\Phi6$ when host bacteria are common. That is, because host density negatively affects the rate and duration of plaque enlargement, the performance of $\Phi6_M$ relative to $\Phi6$ should generally improve with host density, perhaps saturating at a maximum relative fitness for the deleterious $\Phi6_M$ when host densities are very high. To test whether $\Phi6_M$'s fitness improved relative to $\Phi6$ as host density increased in the structured habitat, we used replicated paired-growth assays to estimate fitness of $\Phi6_M$ across 10 different initial host densities (see Methods). Our data (Fig. 4) confirmed that selection against $\Phi6_M$ was weakened under conditions of high host density, echoing the results in Figure 2. A quadratic regression provided the best fit for $\log_{10} W$ values of $\Phi6_M$ as a function of $\log_{10}$ bacterial density ($P < 0.0001$), suggesting that fitness of these phages relative to $\Phi6$ may indeed saturate at $\log_{10} W \approx -0.125$ in the structured habitat.

UNSTRUCTURED HABITATS

Effect of host density on phage titer

To examine whether host density affected phage growth in unstructured habitats, we estimated phage titer (plaque-forming units mL^{-1}) across host densities in a well-mixed liquid. Both genotypes showed titers that increased with initial host density (Fig. 5). An ANCOVA was used to examine effects on $\log_{10}$ phage titer of genotype, $\log_{10}$ host density, and their interaction. We first determined that the interaction was not significant ($F = 1.55$, $P = 0.224$), allowing it to be dropped from the model. Both host density ($F = 22.07$, $P < 0.0001$) and genotype ($F = 18.85$, $P = 0.0002$) had significant effects on viral titer. The positive impact of host density on phage growth likely results from the ease of phage/host encounters in well-mixed fluids; at low host densities phages can kill all sensitive bacteria before they can be replenished, whereas at high host densities the initially rare phage

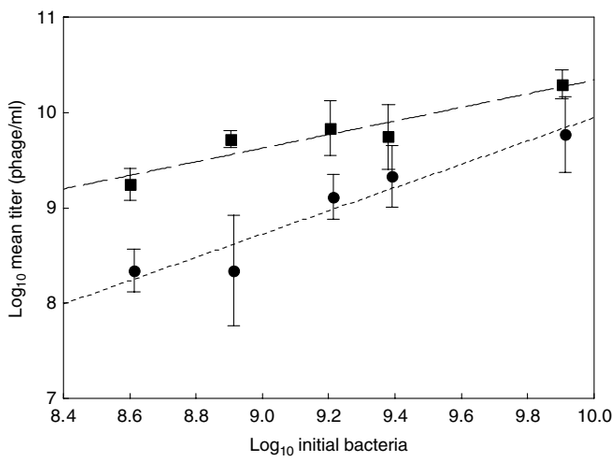

Figure 5. Mean $\log_{10}$ titer of $\Phi6_M$ (filled circles $\pm$ SE) and of $\Phi6$ (filled squares $\pm$ SE) are increasing functions of $\log_{10}$ initial host density in unstructured habitats. Lines are least square regressions. See text for details and statistical analyses.

impact the host population less severely, allowing bacterial growth that facilitates subsequent infections. However, we note that the overwhelming majority of bacteria in all of our experimental treatments were lysed regardless of initial host density; by 24 h all cultures were visibly "clear" rather than the "cloudy" (turbid) aspect of uninfected bacterial cultures. Thus, for the range of host densities examined, the structured habitat provided refugia for some host bacteria (i.e., ordinary lawn growth occurred in between distinct plaques), whereas the unstructured environment largely did not.

Phage fitness at different host densities

The phage titer results suggested that the relative fitness of the phages should be unaffected by host density in the unstructured habitat. To test this idea, we estimated relative fitness of $\Phi 6_M$ using replicated paired-growth assays at five different initial densities of bacteria (see Methods). As predicted, we found no significant effect of $\log_{10}$ host density on $\log_{10}$ W of $\Phi 6_M$ (Fig. 4; slope = 0.014, $t = 0.68$, $P = 0.51$). Overall, mean $\log_{10}$ W of $\Phi 6_M$ equaled $-0.483 \pm$ SE 0.037 across the treatments. These results sharply contrasted the data obtained in the structured habitat (Fig. 4), and we concluded that fitness of the deleterious mutant was more variable when habitat structure was imposed.

PHAGE "LIFE-HISTORY" PARAMETERS

To examine how the fitness advantage of Φ6 was afforded by differences in life-history parameters (Abedon 1989; Abedon et al. 2001; Bull et al. 2004), we conducted classic single-burst experiments ($N = 3$) in liquid that estimated latent period (L), maximal growth rate (m) during the rise period, and burst size (B) of a single infectious cycle. These assays were performed at ratios of 4×10^6 phages to 4×10^9 bacteria; assays for the two phage genotypes were conducted independently, but in parallel to avoid confounding block effects. Results (Table 1) showed that Φ6 and $\Phi 6_M$ did not differ in maximal growth rate or latent period (m: $t_s = -1.345$, df = 4, $P = 0.249$; L: $t_s = 2.234$, df = 4, $P = 0.0892$). By contrast, Φ6 had a significant advantage in burst size ($t_s = -2.856$, df = 4, $P = 0.0461$), suggesting that this trait accounted for the genotype's fitness advantage in the unstructured habitat. Although the burst assays were conducted in liquid at a single phage-to-host ratio, they should be generally useful for contrasting life-history parameters of the viruses whenever phage genotypes are predominantly infecting different cells, as in all of our experiments. We therefore cautiously suggest that the productivity difference between the viruses also drives the results obtained in semisolid agar.

EFFECT OF HOST DENSITY ON PHAGE ATTACHMENT RATE

One parameter sometimes explored in models of plaque enlargement is the rate at which phages bind to host cells (k, attachment rate; Abedon and Culler 2007; Abedon and Yin 2008). All else being equal, a phage with a higher k should be capable of attaching to more hosts within a given time interval (i.e., should experience a shorter host "handling time"; Stopar and Abedon 2008). We therefore considered that k might differ between the viruses, and that this difference may vary across the host densities in our experiments. We measured attachment rates for Φ6 and $\Phi 6_M$ at three initial host densities (see Methods), with threefold replication. The resulting measurements (Table 1) were similar to previous estimates for Φ6 genotypes (Dennehy and Turner 2004). We found that k did not differ according to genotype (ANCOVA; $F = 0.035$, $P = 0.85$) or host density ($F = 0.521$, $P = 0.61$), strongly suggesting that variable attachment rates across different host densities did not contribute to the phenotypic differences seen between $\Phi 6_M$ and Φ6 in our experiments (i.e., Figs. 1, 2, 4).

Discussion

OVERVIEW OF PHAGE POPULATION GROWTH

In unstructured (e.g., broth) habitats, phage should grow faster with increasing cell density, although the rate may become slow at very high bacterial densities because phage transmission time between cells can become shorter than virus generation time (Abedon et al. 2001), and stationary-phase cells are generally less competent for infection. Thus, the total phage production should equal the number of infected bacteria multiplied by the number of

Table 1. Comparison of growth parameters for Φ6 and $\Phi 6_M$ phages obtained from single-step growth and adsorption assays with threefold replication. Entries are mean values ± standard errors. Only attachment rate was measured at three different densities of host bacteria. See text for details and statistical analyses.

		Φ6	$\Phi 6_M$
Attachment rate (k)	High density	4.73×10^{-11} ($\pm1.5594\times10^{-11}$)	5.03×10^{-11}($\pm2.5312\times10^{-11}$)
	Medium density	2.59×10^{-11} ($\pm1.1064\times10^{-11}$)	3.77×10^{-11} ($\pm1.3174\times10^{-11}$)
	Low density	4.19×10^{-11} ($\pm1.8585\times10^{-11}$)	3.49×10^{-11} ($\pm1.3071\times10^{-11}$)
Latent period (L)		76.00 min (±3.606)	83.67 min (±2.728)
Growth rate (m)		0.078 ln phage min^{-1} (±0.003)	0.073 ln phage min^{-1} (±0.002)
Burst size (B)		126.220 phages (±9.977)	95.533 phages (±3.967)

phages produced per infection (i.e., burst size), minus those that initiated the culture (Abedon 1994; Turner and Duffy 2008).

Structured (e.g., agar) habitats are often used to study phage, but here the effects of cell density on phage growth can be more difficult to elucidate. Because phages are submicroscopic, the most convenient method to "observe" them is through lesions that form when phage are allowed to infect bacterial lawns; a single phage that infects a cell within the lawn produces progeny that diffuse outward, killing all bacteria in the immediately surrounding region (You and Yin 1999; Abedon and Culler 2007; Abedon and Yin 2008). The result is a visible hole (or plaque) in an otherwise cloudy lawn of bacteria. Prior to maturity, a growing plaque resembles a traveling-wave epidemic (You and Yin 1999; Abedon and Culler 2007; Abedon and Yin 2008), where the resulting wavefront may be described as a reaction–diffusion process: net movement outward at the periphery of a plaque occurs through virion diffusion, whereas infection (the "reaction" in reaction-diffusion) feeds this movement by producing additional virions. Because bacteria are nonmotile during plaque formation, infection in this reaction-diffusion process represents a time when a phage is no longer moving outward. Theory suggests that bacteria density can impact the rates of plaque spread (reviewed by Abedon and Culler 2007). However, although the impact of initial bacterial density on plaque size and duration of growth is relatively simple, its impact on plaque wavefront velocity is more subtle.

Host density should impact phage spread within plaques, but for reasons different than those affecting growth in broth. In particular, at relatively low host densities, phages must diffuse further to encounter and then infect cells, similar to a predator that travels longer distances to find rare prey. If hosts are sufficiently rare so that phage production rather than diffusion rate limits phage advancement, then greater bacterial densities should increase the rate of plaque growth. However, bacterial density may eventually reach a point where plaque wavefront velocity and growth begin to level out or even decline, because phage diffusion becomes the limiting factor. Although the theory is controversial, phages may become increasingly tied up in bacterial infections (or in nonadsorbing phage/cell interactions) at the plaque periphery when host density is high (Yin and McCaskill 1992; Ortega-Cejas et al. 2004). This negative impact of host density on rates of plaque growth is debated, but at least one empirical study (Mayr-Harting 1958) showed that plaque wavefront velocity declines with increasing host density, and there is evidence that above a threshold lawn density plaque size is inversely proportional to bacterial abundance (Mayr-Harting 1958; Burch and Chao 2004).

HOST DENSITY AFFECTS PLAQUE SIZE

We observed that increasing host density negatively impacted the epidemic spread (plaque size) of two phage Φ6 genotypes. These data echoed a previous study by Burch and Chao (2004), which also showed a general reduction in plaque sizes when Φ6 viruses grew at elevated host densities. One notable difference between the two studies is that Burch and Chao (2004) examined plaque sizes at initial host densities spanning approximately 5×10^6 and 5×10^{10} cells mL^{-1}, whereas we conducted a greater variety of viral growth and relative fitness assays over a narrower range: $\sim 2.5 \times 10^7$ to 4×10^9 cells mL^{-1}. For this reason, although they observed maximum mean plaque sizes at an intermediate host density of $\sim 10^8$ cells mL^{-1} (see Fig. 4; Burch and Chao 2004), we showed only a decline in mean plaque sizes from a presumably similar maximum (Fig. 1).

We infer that these declines with higher initial bacterial densities are due to increasing host availability interfering with phage progeny diffusion and/or that high initial host densities cause bacteria to enter stationary phase (i.e., achieve carrying capacity) earlier in the 24 h experiments, thereby limiting the opportunity for plaque diameters to increase. Although we did not examine plaque growth at various host densities below 10^8 cells mL^{-1} (i.e., conditions in which plaque size should decline from the maximum; Burch and Chao 2004), we can posit two plausible, but not mutually exclusive hypotheses for why plaque sizes might decline at reduced initial bacterial density. First, with lower bacterial densities phage numbers, rather than rates of phage diffusion, may become limiting in terms of controlling plaque wavefront velocity, resulting in slower plaque growth. Second, low initial host densities should prolong the time of first infection by phages, especially if phages are not preadsorbed to bacteria prior to plating, resulting in greater variance in plaque size with the smaller plaques reducing the plaque size average.

MINIMAL CHANGE IN RELATIVE PLAQUE SIZE

Burch and Chao (2004) observed less-variable plaque sizes for low-fitness Φ6 genotypes, and concluded that these viruses showed environmental robustness (i.e., phenotypic constancy across host density). We similarly observed less variability in mean plaque size across the host density treatments for a reduced-fitness Φ6 strain, but we offer a different explanation for the phenomenon. The absolute change in mean plaque size across host densities is greater for Φ6 (Δ mean plaque size $= 1.63$ mm^2) than for $\Phi 6_M$ (Δ mean plaque size $= 1.08$ mm^2). However, the percent change in mean plaque size for the two phages is very similar: 67.27% for Φ6 versus 73.67% for $\Phi 6_M$. Thus, plaque sizes for the two viruses declined proportionally as host density increased, strongly suggesting the differential change in absolute plaque size across host densities is a geometric phenomenon and not due to genetic differences in environmental robustness. Further support for our interpretation is a regression of the ratios of mean plaque sizes for the two viruses versus $\log_{10}$ host density, which showed a slope not significantly different from zero (slope $= -0.118$, $t = 1.42$, $P = 0.29$).

BACTERIOPHAGE LIFE-HISTORY PARAMETERS

The only measurable difference in life-history parameters between Φ6 and $\Phi6_M$ was burst size (see Table 1). However, the difference in latent period was borderline statistically significant. Latent period length and burst size are inextricably related; longer latent periods provide more time for phage reproduction within a cell, thus increasing burst size (Abedon 1989; Abedon et al. 2001, 2003; Wang 2006). It is possible that a statistical difference in latent period might have obtained with increased experimental replication of burst assays. Regardless the two results reinforce each other, and imply that Φ6 is the more-productive phage. In the unstructured habitat, this advantage was expressed universally regardless of initial host density. By contrast, in structured habitats, restricted phage dispersal may have magnified the importance of local productivity, thus allowing the relative fitness of the less-productive $\Phi6_M$ virus to improve as host density increased.

CONSTANT PLAQUE PRODUCTIVITY: A PARADOX

Our observed difference in burst size between Φ6 and $\Phi6_M$ was satisfying because intuition, as well as plaque modeling (Abedon and Culler 2007), suggests that the more-productive virus should make larger plaques, as we observed. However, it is unclear why Φ6 produced constant PPP across host densities, whereas $\Phi6_M$ showed greater plaque productivity as host density increased (Fig. 2). Larger plaque sizes do not necessarily imply greater phage productivity because plaque size is indicative of the disappearance of bacteria, not increased phage numbers; however, we at least expected the relationship between plaque size and productivity to be consistent between the two strains.

The constant plaque productivity of Φ6 is unexpected for two reasons. First, data indicated no change in PPP for Φ6 as initial bacterial density increased. However, the simultaneous decline in plaque size indicated that the number of phage per unit size of the plaque (i.e., phage density) rose as plaque size decreased. Second, whatever phenomenon led to an increase in the particle density within wild-type plaques must have operated at an even greater level in $\Phi6_M$ for phage PPP to increase with initial bacterial density. To address which phenomenon might underlie these confusing observations, we considered how plaques might vary as a function of initial bacterial density.

One possibility is that the wide separation of bacteria at low initial densities should less effectively support phage production within the resulting plaques. However, over time these bacterial densities should increase, perhaps resulting in very similar bacterial numbers at the end of plaque formation, regardless of initial host densities. Because the majority of bacteria infection during plaque development is thought to occur during the last 20% of plaque formation (Kaplan et al. 1981), early events in plaque formation should make little difference. If the duration of plaque development were instead constant, then few infections early on would be relevant, except that plaque formation should last longer when initial bacterial densities are smaller, thereby compensating if phage production starts slowly.

We prefer a second possible explanation: bacteria may differ physiologically over space depending on their initial densities. This phenomenon may be attributed to the fact that bacteria form microcolonies within a lawn, and microcolony size depends on initial bacterial density (Kaplan et al. 1981). Lower initial inocula lead to larger microcolony sizes. This outcome makes intuitive sense if we assume that microcolonies are spheres that are packed within a constant volume (the top-agar layer). Each microcolony is initiated by a single bacterial cell seeded in the top agar. Thus, if fewer bacteria are seeded, then microcolonies must grow to a larger size to attain the same cumulative volume.

Large microcolonies contain relatively fewer outer-surface bacteria with access to oxygen and nutrients, and with relatively unobstructed diffusion of wastes. For these reasons, large microcolonies may contain lower numbers of bacteria that are competent for phage infection. Thus, the final 20% of infections at low initial bacteria densities likely result in reduced burst sizes per cell (due to the larger microcolony size) and, therefore, fewer PPP. This effect could be substantial with a *Pseudomonas* host given that it is an obligate aerobe, and that bacteria in the center of a *Pseudomonas* microcolony may be particularly physiologically inappropriate for phage infection. To summarize, greater input of bacteria into a habitat may lead to smaller microcolonies that contain greater numbers of bacteria competent for phage infection, and this may lead to better phage growth especially later during plaque development, when the majority bacterial infections occur.

Why does the less-fit strain display even greater increase in phage density as plaques form under greater bacterial densities? We speculate that the presumptive poorer host physiology with larger microcolony size has a greater impact on $\Phi6_M$ relative to wild-type Φ6. Alternatively, $\Phi6_M$ may be less able to efficiently penetrate into larger microcolonies, resulting in fractionally fewer bacteria infected within the confines of the plaque, rather than fewer phage produced per bacterium infected. Regardless of the mechanism, it need not be powerful to give rise to the relatively small differences we observed (Fig. 2). Our fitness assays in the structured habitat, which were based on comparative estimates of particles produced per plaque, also show this improvement in relative fitness of $\Phi6_M$ as host density increased (Fig. 4).

DIFFERENCES IN VIRUS PRODUCTIVITY AND PRUDENCE

Phage $\Phi6_M$, but not Φ6, produced greater PPP as cell density increased. Thus, compared with $\Phi6_M$, Φ6 improved less in its local growth as hosts became numerous. Perhaps this result is a "cost" of being successful. Due to its inherent burst size advantage, Φ6 has a generally greater capacity to kill host bacteria in the lawn

before they can be replenished by cell division. Although favorable at low host densities, this "less prudent" strategy leads to reduced benefits for Φ6 when host cells are numerous. That is, when hosts in the structured habitat are scarce, creating many offspring is advantageous because this enhances the probability of encountering rare hosts. But when hosts are common, the faster epidemic spread of Φ6 may quickly overwhelm the locally available hosts as the bacterial lawn approaches carrying capacity and becomes noncompetent for infection. That is, the highly productive phages may eliminate available hosts before they have a chance to reproduce, thus reducing the total number of hosts available for infection.

We earlier reported a similar result in this phage system (Dennehy et al. 2006): increasing the size of the initial phage inoculum in a structured habitat did not translate into greater overall productivity, and even led to declines in productivity at very high inocula. Analogous disadvantages of high virulence are suggested in true disease systems, such as variants of ebola virus whose epidemics seem to "burn out" because rates of human fatality exceed the input of susceptible host individuals needed to locally sustain the pathogen (Pourrut et al. 2005).

Other recent work demonstrates that relative success of parasite genotypes is influenced by the extent of habitat structure. For example, Boots and Mealor (2007) used laboratory microcosms to study evolution of a granulosis virus that is directly transmitted between moth larvae hosts. They predicted that parasite infectivity (i.e., transmissibility between infected and susceptible hosts) should decrease when host movement is restricted because this reduces the probability that infected hosts will become surrounded by other infected hosts (i.e., "self-shading," which negatively impacts transmission). As predicted, greater habitat viscosity caused caterpillars to congregate, in turn selecting for low-infectivity, prudent viruses (Boots and Mealor 2007). The authors noted that, in other disease systems, productivity (i.e., the number of infective particles released into the habitat), rather than infectivity, may be more crucial for transmission.

A separate experiment by Kerr et al. (2006) showed that restricted movement impacted the evolution of prudent strategies in phage T4 infecting bacteria. Kerr et al. took advantage of a high-throughput liquid-handling robot to move phages and hosts between wells of 96-well microtiter plates in which initial well state (i.e., presence of bacteria and/or phages) was determined by simulation. When phage and bacteria dispersal was manipulated to occur only locally (i.e., to neighboring wells of a microtiter plate), the viruses quickly killed all the bacteria in the wells. This scenario selected for prudent phages, whose reduced productivity allowed greater host reproduction, thereby increasing the phage yield from a well and the probability of phage transmission. By contrast, when phages and bacteria could disperse to any well, increased phage productivity was positively selected because it increased the likelihood of phages encountering a well containing hosts. To summarize, Kerr et al.'s experiment manipulated migration to show that restricted dispersal benefited prudent phages, whereas our work suggested that unmanipulated (but restricted) dispersal in agar is also sufficient to benefit prudent viruses. In other words, the absence of direct competition between phage types growing in different plaques reduced the fitness costs that are otherwise seen when phages compete head to head for the same bacteria, as in well-mixed broth (Abedon et al. 2003).

IMPACT OF HOST DENSITY AND HABITAT STRUCTURE ON PHAGE SELECTION

Both PPP and plaque size increased for $\Phi 6_M$ when initial host density in the structured habitat increased. By contrast, $\Phi 6_M$ produced consistently low titers at all host densities when the two genotypes were grown in well-mixed liquids. For these reasons, the fitness of $\Phi 6_M$ relative to Φ6 was, on average, more variable in the structured habitat across the bacterial densities examined, sometimes causing the strength of selection against $\Phi 6_M$ to be reduced. In a study using the same microbes, Froissart et al. (2004) showed that the strength of selection against deleterious Φ6 mutants can vary in agar habitats, but the selection coefficients depended on the degree of coinfection experienced by the phage genotypes. In particular, the experiment showed that the process of purifying selection (i.e., removal of deleterious alleles) in virus populations occurs more slowly when phages are cultured at high multiplicities, where the phages greatly outnumber their host bacteria and most cells are multiply infected. As explanation, Froissart et al. cited the importance of complementation, where the deleterious genes of virus mutants are masked by complementary genes of superior genotypes within the cell.

The current study was designed to minimize coinfection, and thereby complementation, between different phage genotypes. Thus, we demonstrated an additional mechanism, habitat structure, which can lead to weakened selection against deleterious mutants. Although we designed our experiments to minimize coinfection involving different genotypes, the possibility exists that late in the 24 h assays the ratio of phages to host bacteria changed so that some coinfection took place. However, if this transition occurred we do not believe that it impacted our interpretation of the experiments. In the structured habitat, the phages formed nonoverlapping plaques, such that any coinfection would have occurred within a plaque, involving identical parent genotypes and, hence, no complementation.

Our results also relate to an earlier study involving wild isolates of Cystoviruses, the virus family to which Φ6 is classified. Silander et al. (2005) isolated wild samples of Cystoviruses and subjected these clones to phylogenetic and population genetic analyses to infer population structure in natural phage communities. The results suggested that coinfection occurs often in

terrestrial phage communities, such that genetic variants created through genetic exchange (hybrids) may rival the frequency of those appearing through spontaneous mutation alone. Thus, the data hint that hybrid genotypes are not strongly selected against in wild populations, and intracellular mechanisms such as complementation may foster persistence of hybrids even if they are of relatively low fitness. The current study offers an additional mechanism, suggesting that differing densities of available hosts may also contribute to persistence of phenotypically inferior hybrids or deleterious mutants in phage populations, provided that these wild populations are growing in structured habitats. Indeed Φ6 and its hosts commonly do grow in structured habitats: plant leaf surfaces (Silander et al. 2005). No study has attempted the difficult task of measuring fluctuations in bacterial densities experienced by Cystoviruses in the wild, so we are left to speculate about the relative roles of host density/habitat structure versus coinfection in modulating strength of selection against deleterious mutants. But it is likely that all phage populations should occasionally experience bacterial populations of differing densities in the wild. Therefore, we cautiously suggest that this phenomenon should produce differing selection intensity experienced by phage genotypes, provided some habitat structure also exists.

ACKNOWLEDGMENTS

We thank N. Friedenberg, M. Travisano, the members of the Turner Lab and four anonymous reviewers for suggested improvements to the manuscript. B. Alto lent statistical expertise, R. Montville provided excellent technical help, J. Pease assisted with some of the experiments, and L. Mindich (Public Health Research Institute, Newark, NJ) and L. Chao (University of California, San Diego, CA) kindly provided biological material. This study was funded by the U.S. National Science Foundation: grant #DEB-04–52163 to PET and postdoctoral fellowship #DBI-03–10205 to JJD.

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Associate Editor: M. Travisano