followed by Cy3-labelled goat anti-rabbit (1:700; Jackson Immunoresearch), Cy2-labelled donkey anti-guinea-pig (1:100; Jackson) and/or FITC-labelled horse anti-mouse (1:500; Vector) antibodies. For size-distribution studies, immunofluorescently stained sections double-labelled with anti-NeuN[29] were analysed using NIH image software. Glucose oxidase/nickel-enhanced diaminobenzidine immunostaining of spinal cord sections was performed using anti-VRL1 (0.83 $\mu g\,ml^{-1}$) as described[5].

Primary cultures prepared from adult rat DRG[30] were incubated overnight (37 °C, 5% CO_2) in medium containing nerve growth factor (100 $ng\,ml^{-1}$) and fixed with 10% formalin in 0.1 M phosphate buffer. For size-distribution studies, cells were stained with anti-VR1 or anti-VRL1 IgG (166 $ng\,ml^{-1}$), detected with diaminobenzidine–peroxidase (Vector), and analysed as above.

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Prisoner's dilemma in an RNA virus

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The evolution of competitive interactions among viruses[1] was studied in the RNA phage φ6 at high and low multiplicities of infection (that is, at high and low ratios of infecting phage to host cells). At high multiplicities, many phage infect and reproduce in the same host cell, whereas at low multiplicities the viruses reproduce mainly as clones. An unexpected result of this study[1] was that phage grown at high rates of co-infection increased in fitness initially, but then evolved lowered fitness. Here we show that the fitness of the high-multiplicity phage relative to their ancestors generates a pay-off matrix conforming to the prisoner's dilemma strategy of game theory[2,3]. In this strategy, defection (selfishness) evolves, despite the greater fitness pay-off that would result if all players were to cooperate. Viral cooperation and defection can be defined as, respectively, the manufacturing and sequestering of diffusible (shared) intracellular products. Because the low-multiplicity phage did not evolve lowered fitness, we attribute the evolution of selfishness to the lack of clonal structure and the mixing of unrelated genotypes at high multiplicity[4–6].

Evolutionary game theory predicts the outcome of pairwise contests between players that use conflicting strategies[3]. If a population of individuals playing one strategy cannot be invaded by mutants playing any other strategy, the former becomes the evolutionarily stable strategy. But if no single strategy is able to resist invaders, a polymorphic equilibrium with both strategies ensues. Whether a single strategy or a polymorphic equilibrium evolves depends on the fitness pay-offs to the players. In a contest between the strategies of cooperation and defection, the resulting 2×2 pay-off matrix is described by three variables (Fig. 1a). When a pair of cooperators interact, their individual fitness has a value of one. When a cooperator and a defector are paired, the cooperator is exploited and its fitness is decreased by s_1, whereas the defector benefits and its fitness is increased by s_2. When two defectors interact, they suffer from not having anyone to exploit and pay a cost c. If $(1 - c) < (1 - s_1)$ a polymorphic equilibrium results. If $(1 - c) > (1 - s_1)$ the population evolves to be 100% defectors. The latter is evolutionarily paradoxical, and termed the prisoner's dilemma, because a population composed of defectors has a lower fitness than one containing only cooperators. Although the prisoner's dilemma is clearly of evolutionary importance, it is difficult in most biological systems to measure the pay-off values associated with it[7–9] and most examples are theoretical[10].

Viral evolution offers a unique opportunity to study the prisoner's dilemma because co-infection of the same host cell by more than one virus creates conflicts[1,11–13] similar to those assumed in game theory, and ancestral genotypes can often be retrieved for reconstructing the pay-off matrix. The manufacture of diffusible and hence shared intracellular products by viruses co-infecting the same cell allows for the strategies of cooperation and defection. A viral genotype that synthesizes larger quantities of products is effectively a cooperator. In contrast, a genotype that synthesizes less but specializes in sequestering a larger share of the products is a defector. The often observed evolution of defective interfering particles[14] in viruses supports this interpretation. The particles are coded by viral RNAs that have lost most or all of their protein-coding sequences,

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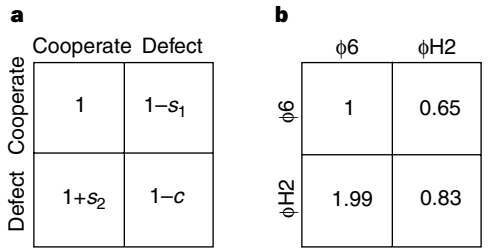

Figure 1 Expected and observed fitness values for a game in which opponents use conflicting strategies of cooperation and defection. **a**, Generalized pay-off matrix where entries represent the fitness to an individual adopting the strategy on the left, if the opponent adopts the strategy above. Defectors gain a fitness advantage $(1+s_2)$ that allows them to invade a population of cooperators. If the cost of defection is too strong, $(1-c)<(1-s_1)$, cooperators may also invade and the two strategies are driven to a stable polymorphism. The prisoner's dilemma occurs if it always pays to be selfish, $(1-c)>(1-s_1)$; defection sweeps through the population despite the greater fitness pay-off that would result had all individuals cooperated. **b**, Realized pay-off matrix for the evolved high MOI phage ϕH2 relative to its ancestor ϕ6 reveals evolution of an evolutionarily stable strategy conforming to the prisoner's dilemma. Observed fitness values indicate that the lowest pay-off is to the ancestor during co-infection. Thus, the evolutionarily stable strategy is to defect even though a higher pay-off occurs when phage cooperate.

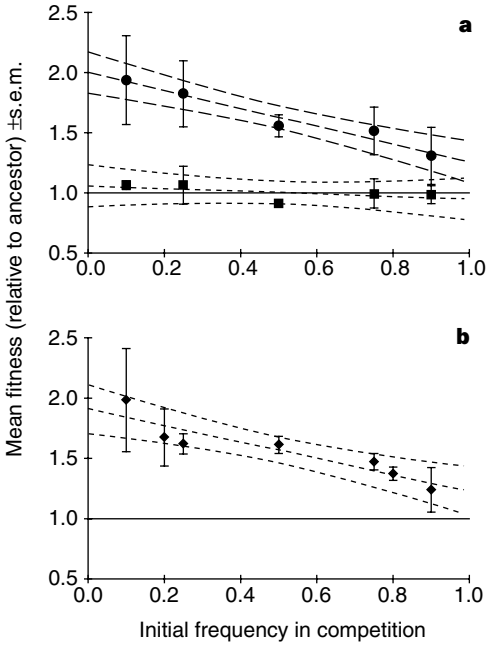

Figure 2 The fitness of derived high MOI phage relative to the ancestor is a decreasing function of initial frequency in competition. **a**, Evolved phage ϕH2 and a genetically marked clone of the ancestor, ϕ6, were competed at five initial frequencies of ϕH2 (0.1, 0.25, 0.5, 0.75, 0.9) at MOI = 5 with fourfold replication. Linear regression analysis shows that the fitness of ϕH2 (circles) is dependent upon its initial frequency in competition (slope = −0.7381, $t_s = -8.117$, d.f. = 3, $P = 0.0039$), whereas a control that measures fitness of ϕ6 (squares) relative to the marked clone is independent of initial frequency (see Methods for statistics). Values are the means ± s.e.m. Dashed lines indicating the 95% confidence interval about each regression line are included to show that mean fitness of ϕH2 exceeds 1.0 at all initial frequencies. Phage ϕH2 is ϕS2 from a previous study[1]. **b**, Experiments where a different evolved clone, ϕH1, and the marked ancestor were competed at seven initial frequencies of ϕH1 (0.1, 0.2, 0.25, 0.5, 0.75, 0.8, 0.9) at MOI = 5 with fourfold replication. Once again, linear regression analysis shows that the fitness of ϕH1 (diamonds) is dependent upon its initial frequency in competition (slope = −0.6789, $t_s = -5.128$, d.f. = 5, $P = 0.0037$), and exceeds 1.0 at all initial frequencies.

and are thus unable to reproduce in the absence of complete viruses. This inability defines the defective nature of defective interfering particles but they are also functionally defectors (in the game theory sense) because they have evolved a variety of mechanisms to gain an intracellular fitness advantage in the presence of complete viruses. Their smaller size allows some particles to replicate faster, whereas others skip unnecessary transcription steps or are preferentially processed because they feature extra sequences recognized by encapsidation and replication enzymes[14–16]. By providing the necessary enzymes for defective interfering particles, complete viruses act functionally as cooperators.

Because a population composed only of defective interfering particles is unable to reproduce and $(1-c)$ equals zero, the evolution of the particles results in a polymorphic equilibrium and does not constitute a case of the prisoner's dilemma. Thus it becomes important to determine whether complete (non-defective interfering) viruses can also evolve quantitatively different strategies on a continuum of cooperation and defection, and whether any of the resulting strategies conform to the prisoner's dilemma. A previous study allowed replicate populations of ϕ6 to evolve at high and low multiplicities-of-infection (MOI)[1]. Data showed that phage cultured at high MOI (but not low MOI) gain an added advantage during co-infection, indicating that these viruses evolved a defection strategy for intracellular competition. Further results indicated that in environments where high MOI phage become fixed, and hence intracellular competition with other genotypes is removed, these viruses exhibit evolution of lowered fitness. Because the evolution of lowered fitness in a population of defectors is expected from the prisoner's dilemma, we have investigated further whether game theory could be used to interpret this surprising result.

Although the prisoner's dilemma leads to fixation (a population playing a single strategy), its effects should still be frequency dependent because defectors gain their greatest fitness advantage when rare and interacting primarily with cooperators (Fig. 1a). Thus, we first sought evidence of whether fitness of the evolved high MOI phage was dependent on their initial frequency in competition. We isolated two clones (ϕH1 and ϕH2) at random from separate populations evolved at high MOI. The fitness of each clone was measured relative to the ancestor, ϕ6, at an MOI of five that reflects the high degree of co-infection experienced by ϕH1 and ϕH2 during their evolution. A host-range mutation[17] was introduced into ϕ6 to serve as a genetic marker that differentiates the ancestor during fitness assays. The results (Fig. 2) clearly show that the fitness of ϕH1 and ϕH2 is a decreasing function of each clone's initial frequency, whereas controls involving ϕ6 and the marked ancestor demonstrate that frequency-dependence is not driven by the host-range marker. More importantly, these results also demonstrate that for frequencies approaching 1.0 the mean fitness of ϕH1 and ϕH2 exceeds unity. A fitness value equal to 1.0 would have implied that the fitness of the high MOI phage was equal to that of ϕ6. Rather, the fitness of ϕH1 and ϕH2 exceeds 1.0 at all initial frequencies despite the negative effect of frequency on fitness, demonstrating that ϕH1 and ϕH2 will achieve fixation as required by the prisoner's dilemma.

The support for the prisoner's dilemma in Fig. 2 is best illustrated when these data are used to estimate s_1, s_2 and c in our pay-off matrix (Fig. 1a). Because the fitness data for ϕH1 and ϕH2 are virtually identical, we chose to explore the prisoner's dilemma hypothesis further using only ϕH2 (Fig. 2a). Fitness was measured at a high MOI, where most phage reproduce in co-infected cells. At low initial frequencies of ϕH2, pure co-infections containing only ϕH2 are rare and the fitness of ϕH2 is primarily determined by mixed co-infections that also contain ϕ6. Thus, the fitness of ϕH2 at low frequencies is equal to $(1+s_2)$. It follows that ϕ6 is very abundant at low frequencies of ϕH2 and most co-infection events will occur through the ancestor alone. If the fitness of ϕ6 in a pure

co-infection is defined to be one, then the y-intercept of the regression line in Fig. 2a equals $(1 + s_2)/1$, which provides the estimate of $(1 + s_2) = 1.99$. By the same logic, the fitnesses of ϕH2 and ϕ6 at high initial frequencies of ϕH2 are $(1 - c)$ and $(1 - s_1)$, whereby the ratio $(1 - c)/(1 - s_1)$ is estimated by the extrapolated fitness value in Fig. 2a at a frequency of 1.0. Because the extrapolated value of 1.28 is greater than 1.0, $(1 - c) > (1 - s_1)$ as required by the prisoner's dilemma.

However, to demonstrate simply that $(1 - c) > (1 - s_1)$ does not provide definitive support for the prisoner's dilemma. Rather, to complete the pay-off matrix, either $(1 - c)$ or $(1 - s_1)$ must be estimated separately and we devised an experiment that measures $(1 - c)$. Adsorption is the step in phage infection that involves attachment and entry of viruses into the bacterial cell[18]. We modified our fitness assay so that each phage was allowed to adsorb separately at a high MOI, and then mixed the adsorbed phage (each at a frequency of 0.5) just before the fitness assay. This approach differs from our previous experiments (Fig. 2) in which the phage were mixed before adsorption. Thus, whereas previous assays contained cells co-infected by both ϕH2 and ϕ6, the present assay contains only cells infected with either ϕH2 or ϕ6 genotypes. Without co-infection, the fitness of ϕH2 is $(1 - c)$ and that of ϕ6 is 1 (Fig. 1a). Results showed $(1 - c)$ to be 0.830 ± 0.051 (mean ± s.e.m., $n = 10$), in which case $(1 - s_1) = 0.83/1.28 = 0.65$. Note that in our previous assays involving mixed infections the fitness of ϕH2 is much higher at a frequency of 0.5 (compare with Fig. 2a).

Substitution of parameter estimates into our pay-off matrix allows us to reconstruct the evolution of viral fitness at high MOI (Fig. 1b). The defection strategy exhibited by ϕH2 affords a large fitness advantage relative to the ancestor during co-infection, allowing this mutant to invade when rare. As ϕH2 increases in frequency its intracellular interaction with other genotypes becomes more rare, causing its fitness relative to the ancestor (or other co-operators) to dip below 1.0. This idea is supported by previous data where intrahost competition between viruses was removed[1], and by our current estimates of $(1 - c)$. However, our data also show $(1 - c) > (1 - s_1)$, indicating the lowest fitness pay-off is to the ancestor when ϕH2 is very common. Thus, it always pays to be selfish and the defection strategy shown by ϕH2 is able to sweep through the population. Because all genotypes wind up in the lower right of the matrix despite its inherently low fitness pay-off, this clearly conforms to the prisoner's dilemma.

One way for evolving populations to escape the prisoner's dilemma is through kin selection (but see ref. 19 for an alternative mechanism). During intrahost reproduction, the relatedness of parasite progeny selects for different strategies to use limited host resources[4,6,20,21]. In single infections, either competition is absent or selection favours the evolution of cooperation among closely related individuals. In contrast, mixing of unrelated genotypes favours defection to exploit resources selfishly. Therefore, evolution of parasite strategies during intrahost competition can be viewed as a problem of kin selection[5,22]. We allowed the evolution of a single ancestral virus in two environments where degree of progeny relatedness differed[1]. At high MOI, strong intracellular competition occurred between distantly related individuals, whereas at low MOI competition was either absent or occurred among closely related kin. Thus, our findings that evolution of phage at high MOI results in prisoner's dilemma can be attributed to the absence of clonal structure at high MOI. Lack of clonal structure leads to the evolution of selfishness, but the traditional view is that selfishness evolves through more rapid exploitation of host resources, generally resulting in the evolution of increased parasite virulence and often a concomitant shortened lifespan of the host[23,24]. Our study shows an alternative pathway for selfishness to evolve, that is, viruses gain an advantage by sequestering gene products of co-infecting genotypes, which does not necessarily lead to an increase in virulence. Our data show that fitness trade-offs consistent with the prisoner's dilemma and other game theoretic models readily evolve in biological systems as simple as viruses. Furthermore, the prisoner's dilemma provides a clear case for game theory to challenge the idea that natural selection should always lead to a fitness increase[25]. □

Methods

Culture conditions and fitness assays. Bacterial cultures and phage lysates were grown, incubated and diluted at 25 °C using LC broth and agar[26]. To measure fitness, a genotype was mixed at a defined ratio with a genetically marked clone. The mixture was then allowed 40 min adsorption to *Pseudomonas phaseolicola* at MOI = 5, where greater than 99% of phage are adsorbed by 40 min[27]. Before cells had burst[27], the diluted mixture was plated on LC agar with a *P. phaseolicola* lawn, and then incubated for 24 h. The resulting ~500 plaques on the plate were then collected[26] and filtered (0.22 μm Durapore, Millipore) to remove bacteria. Some fitness assays allowed separate adsorption for competitors. The marker used was a point mutation on the medium segment that allowed growth on the alternative host *P. pseudocaligenes*[17]. Ratio of phages in the starting mixture (R_0) and that in the collected lysate (R_1) were monitored by plating on mixed lawns of *P. phaseolicola* and *P. pseudocaligenes* (200:1 ratio), on which wild-type and host-range clones make turbid and clear plaques, respectively. The number of plaques per plate was kept at 500 by diluting the collected phage with LC broth. A maximum of 500 was chosen to minimize overlap, and thus genetic exchange, between plaques. Fitness (W) was defined as the relative change of the ratio wild-type:host-range, or $W = R_1/R_0$. Competitions between the ancestor and a marked clone (Fig. 2a) showed a small deleterious effect to the marker (mean W of ancestor $= 1.0454 \pm 0.041$ s.e.m.; $n = 20$), that did not differ according to initial frequency of the ancestor (linear regression with slope $= -0.1144$, $t_s = -1.202$, d.f. $= 3$, $P = 0.3156$). In addition, similar competitions at a frequency of 0.5 showed a marker effect (mean W of ancestor $= 1.0598 \pm 0.024$ s.e.m.; $n = 12$) that did not differ according to the presence of co-adsorption (independent samples t-test with $t_s = 0.219$, d.f. $= 10$, $P = 0.831$). All fitness values reported were therefore scaled to adjust for the effects of the marker by setting the mean fitness of the ancestral clone to 1.0.

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