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Phage selection for bacterial cheats leads to population decline

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While predators and parasites are known for their effects on bacterial population biology, their impact on the dynamics of bacterial social evolution remains largely unclear. Siderophores are iron-chelating molecules that are key to the survival of certain bacterial species in iron-limited environments, but their production can be subject to cheating by non-producing genotypes. In a selection experiment conducted over approximately 20 bacterial generations and involving 140 populations of the pathogenic bacterium *Pseudomonas aeruginosa* PAO1, we assessed the impact of a lytic phage on competition between siderophore producers and non-producers. We show that the presence of lytic phages favours the non-producing genotype in competition, regardless of whether iron use relies on siderophores. Interestingly, phage pressure resulted in higher siderophore production, which constitutes a cost to the producers and may explain why they were outcompeted by non-producers. By the end of the experiment, however, cheating load reduced the fitness of mixed populations relative to producer monocultures, and only monocultures of producers managed to grow in the presence of phage in situations where siderophores were necessary to access iron. These results suggest that public goods production may be modulated in the presence of natural enemies with consequences for the evolution of social strategies.

1. Introduction

Cooperation is a pervasive phenomenon in biological systems, and despite considerable study, its establishment and maintenance are incompletely understood. Theoretical work starting with Hamilton's seminal papers [1,2] identified a number of key features that promote cooperation (e.g. [3–5]). While many empirical studies have tested theory using social insects and cooperative birds and mammals [6,7], an increasing number have employed microbes, given their rapid evolution and experimental control relative to metazoa (e.g. [8]).

Numerous studies on both metazoan and microbial species show how ecological variables may influence social evolution, including resource supply [9,10], disturbance frequency [7,11] and spatial heterogeneity [12], but also certain inter-individual effects stemming from social strategies [13], interspecific competition [14], and predation and parasitism [15–17]. Yet key evolutionary forces driving collective behaviour and group formation, such as predation and parasitism (e.g. the selfish herd [15,18]), have received limited attention as mediators of microbial social behaviours.

Predators and parasites may either be the basis of social behaviours, such as cooperative defence (e.g. [16,19,20], but see [21]), or constitute a cost that potentially impacts other cooperative behaviours (e.g. resource access and sharing, quorum sensing [19,22,23]). Such costs may differ between individuals adopting different social behaviours, and include energy or time committed to defence or resistance (e.g. [22,24]), or costs associated with trade-offs involved in evolved resistance to enemies [19,20]. Despite their ubiquitousness in nature and demonstrated importance in population ecology and evolutionary biology (e.g. [25,26]), the impacts of natural enemies on the ecology and evolution of microbial cooperation remain largely unexplored.

Natural enemies could potentially impact cooperation through at least three non-mutually exclusive processes: demography, plasticity and selection. First, predators and parasites may have a demographic effect [27], whereby reduced host or prey density either increases cooperation through lower local competition (e.g. [28]) and higher resource supply [10], or decreases cooperation through less efficient signalling [29]. Second, natural enemies may induce plastic changes in their host/prey behaviour either directly through, for example, physical contact or detection of stimuli (e.g. [30,31]), or indirectly (e.g. [26]) by affecting, for example, communication between neighbours [32]. Third, natural enemies may select for resistance that has pleiotropic and epistatic consequences on cooperation [23], or resistance that promotes the emergence of diversity generating mechanisms influencing cooperation. For example, viruses may select for higher mutation rates in certain bacterial populations [23,33] (but see [34]), which could decrease relatedness and favour the emergence of intermediate phenotypes with varying levels of investment in cooperation [12].

What little is known about how natural enemies affect the social evolution of their prey or hosts in experimental settings comes from phage–bacteria systems. Morgan *et al*. [17] investigated the impact of bacteriophage pressure on public goods production in the form of iron-chelating molecules (siderophores) in the bacterium *Pseudomonas fluorescens*. They found that phages prevent the emergence of initially rare non-producer mutants if the fitness gains of resistance mutations exceed those not producing siderophores. This is because numerically dominant producers are more likely to evolve resistance to phage by chance than less numerous non-producers. Their study also demonstrated positive frequency dependence in the relative fitnesses of both producers and non-producers, but empirical investigation of a possible fitness advantage of the more frequent strategy is still lacking.

We investigated the experimental evolution of siderophore (pyoverdin) production in the pathogenic bacterium *Pseudomonas aeruginosa* PAO1 in the presence and absence of a bacteriophage. We compared the effect of phage under two experimental conditions: an 'iron-limited' environment in which the non-producers have access to iron through siderophore production by producers, and an 'iron-rich' environment in which iron availability is not limiting and non-producers do not gain from the presence of producers. To evaluate frequency-dependent interactions, we established mixed populations of *P. aeruginosa*, with different initial frequencies of the two strategy types. We followed the relative fitness of each type and the production of public goods in the form of siderophores. We show that the fitness advantage to non-producers in competition is significantly increased in the presence of phage under both iron conditions. Moreover, we find evidence for negative frequency dependence of the non-producer strategy. Pyoverdin production by producers is significantly higher under phage pressure and provides a possible explanation for the dominance of non-producers in the presence of phage: augmented pyoverdin production comes at an added cost to producers and may constitute additional benefits to non-producers when iron availability is limited. However, by the end of the experiment, whereas in iron-rich environments the advantage to non-producers led to overall growth in mixed populations, in the iron-limited environment the densities of mixed cultures actually decreased. We discuss these findings in the contexts of public goods dynamics and social evolution theory.

2. Material and methods

(a) Strains and culturing conditions

We employed two isogenic strains of *P. aeruginosa* [35] differing in their production of pyoverdin. The wild-type PAO1 (ATCC 15692) is a pyoverdin producer, whereas the mutant strain PAO1ΔpvdD is unable to produce this siderophore. The mutant strain was constructed by the knockout of the non-ribosomal peptide synthetase gene pvdD on the PAO1 wild-type strain [35]. Prior to our experiment, independent replicate colonies of both strains were inoculated in 30 ml microcosms containing 6 ml of King's B medium (KB) [36] and incubated overnight under constant shaking (200 r.p.m.). M9 minimal salt solution was used for all sample dilutions.

We used a stock of LKD16 phage (Podoviridae [37]) amplified from a single plaque. Briefly, the phage plaque was introduced in an exponentially growing bacterial population of the ancestral *P. aeruginosa* PAO1 and incubated at 37°C for 24 h. Then, 10% chloroform was added to the culture to kill the bacteria. After vortexing and centrifugation (13 000 r.p.m. for 4 min), we recovered the phage-containing supernatant and stored it at 4°C. This master stock was used for all experiments.

Evolution experiments were performed in the inner wells of 48-well plates to prevent evaporation. A microcosm consisted of a well containing 1 ml of casamino acids medium (CAA; 5 g Casamino acids, 1.18 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSo_4 \cdot 7H_2O$, per litre; BD Biosciences). Limited iron availability conditions were obtained by supplementing the CAA medium with sodium bicarbonate to a final concentration of 20 mM and 100 µg ml^{-1} of human apotransferrin (Sigma-Aldrich), a strong iron chelator, which binds free iron and prevents non-siderophore-mediated uptake of iron [38]. For high-iron-availability conditions, CAA medium was supplemented with 30 µM $Fe(III)Cl_3$ (ferric chloride, Sigma-Aldrich). To minimize the level of exogenous iron, all the solutions were prepared using millipore water. The 48-well plates were incubated at 37°C under static conditions.

(b) Evolution experiment

We tested the impact of phage pressure on the siderophore production strategy by measuring the densities and relative frequencies of producers and non-producers under two conditions: a limited iron availability situation (hereafter called 'iron-limited' conditions) in which high siderophore production is required for iron acquisition, and a high iron availability situation (hereafter called 'iron-rich' conditions) in which a given amount of iron is directly available. Under the latter conditions, siderophore production is approximately 20 times lower than the former, and a previous study indicates that pyoverdin production ceases completely when iron supplementation is greater than 50 µM [39].

At the beginning of the experiment, iron-limited and iron-rich 1 ml microcosms were inoculated with ca 5×10^5 cells from the overnight cultures. The treatments were each replicated 10 times and consisted of either monocultures (100% producers or 100% non-producers) or mixed cultures (11, 28, 62, 76 and 91% non-producers), each either with or without phage. Half of the replicates were inoculated with ca 5×10^3 particles of LKD16 phage and the other half were supplemented with the same volume of M9 minimum salt solution as a control (5 µl).

Every 24 h, 10% of each population was transferred into fresh microcosms. The experiment was conducted for five transfers (approx. 15–20 bacterial generations), hereafter referred to as T1–T5 (T0 corresponds to the ancestral populations, T1 the first transfer—populations after 24 h—and T6 the end of the experiment—24 h after transfer T5).

At T0, T1 and T6, populations were plated onto KB agar to estimate total densities, and relative frequencies of producers and non-producers. Non-producers can be distinguished from

the producers on KB agar, as the former produce white colonies, whereas the latter are yellow-green. To limit phage predation associated with phage treatments on KB agar, all populations were centrifuged once at 13 000 r.p.m. for 8 min. The supernatant containing phage was then discarded and the pellet resuspended in salt solution before being plated onto KB agar. This method does not remove all phages from the cultures, but decreases the phage-to-bacteria ratio sufficiently to permit accurate counts of colony forming units on Petri dishes.

Growth of each population was estimated by the Malthusian parameter $m = \ln(N_f/N_0)$ [40], with N_0 and N_f being the initial and final densities, respectively. We calculated population growth at T1 and T6 to compare short- and longer-term effects of phage and iron availability.

To express the relative performance of each strain in mixed populations, we calculated the change in relative frequency in non-producers over time as $v = [q_2(1 - q_1)]/[q_1(1 - q_2)]$, where q_1 and q_2 are, respectively, the initial and final proportions of non-producers [41]. When $v > 1$ ($v < 1$), non-producers (producers) increase in frequency.

(c) Phage–bacteria interactions

(i) Bacterial resistance to phage

Resistance was measured as the proportion of bacterial colonies that grow in the presence of phage. Sixty colonies of each strain (either producers or non-producers) were streaked against a line of phage (30 µl) on iron-rich CAA agar Petri dishes. After 24 h of incubation, a colony sample was scored as sensitive to phage if there was clear inhibition of growth; otherwise it was scored as resistant. We used this method to test the resistance of ancestral bacteria to ancestral phage and the resistance of evolved T6 bacteria to their sympatric T6 phage (three replicates).

(ii) Phage fitness in bacterial hosts

Following Wang [42], we define phage fitness as $w = [\ln(P_t/P_0)]/t$, with P_0 and P_t being the densities of phage at times 0 and t after infection, respectively. We introduced phages (approx. 1000 particles ml^{-1}) and exponentially growing bacteria from each of six replicate populations of either producers or non-producers in 3 ml KB in 30 ml microcosms. After 2 h of incubation, we vortexed a 1 ml sample of each population with 10% chloroform to kill bacteria, centrifuged at 13 000 r.p.m. for 4 min and recovered the supernatant to isolate the phage. The resulting phage solution was serially diluted and mixed with bacteria in soft KB agar (6 g l^{-1} agar in KB medium), and 1 ml was poured over a KB agar Petri dish (three replicates per dilution). The Petri dishes were then incubated at room temperature overnight before being examined for plaque counting.

(d) Pyoverdin production

We assayed pyoverdin concentration in each T6 bacterial population by measuring fluorescence intensity (relative fluorescent units, RFU) in each well of the experimental plates at the end of the evolution experiment (i.e. 24 h after the last transfer) with a spectrophotometer (excitation: 400 nm, emission: 460 nm, Fluostar Optima fluorescence microplate reader, BMG Lab Technologies).

As an additional test for the effect of phage on pyoverdin production, we followed bacterial growth and pyoverdin production of producer monocultures (using optical densities as a proxy for growth, and fluorescence as a proxy for pyoverdin quantity) in both iron-limited and iron-rich media for 12 h. Single colonies were inoculated in 48-well plates, and after 6 h of growth, we introduced 10^7 phages into five of the 10 microcosms for each iron condition. The same volume (10 µl) of M9 minimum salt solution was introduced in the control microcosms.

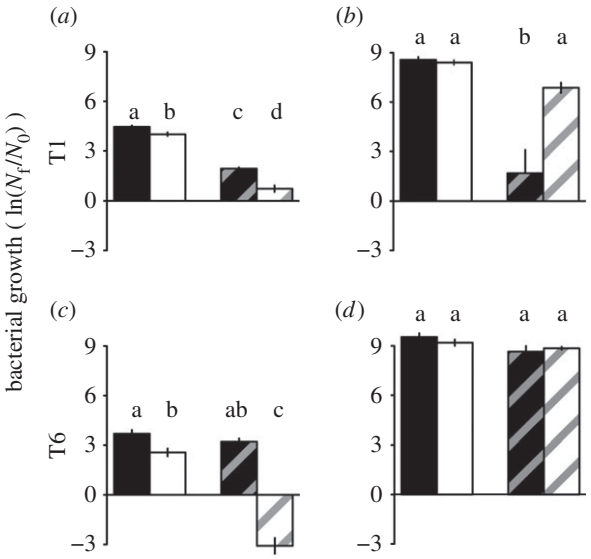

Figure 1. Growth of producers (black histobars) and non-producers (white histobars) in monocultures for (*a*,*b*) the first 24 h and (*c*,*d*) over the five transfers in (*a*,*c*) iron-limited and (*b*,*d*) iron-rich environments. Data are logarithmically transformed. Grey hatched histobars represent populations with phage. Pairwise comparisons made with SNK tests. Means with the same letter do not significantly differ ($p > 0.05$). Bars are standard errors of the mean.

(d) Statistical analysis

All analyses were conducted with R software (R v. 3.1.1; http://www.r-project.org/). Growth of producers and non-producers in monocultures (m) were compared across treatments with full factorial ANOVAs at T1 and T6 using iron availability, phage (presence or absence) and strain as explanatory factors. Pairwise comparisons were made using the Student–Newman–Keuls (SNK) procedure [43] on iron-limited and iron-rich data, separately. Levels of resistance were compared between producers and non-producers using a mixed-effects model with replicate as a random factor. Relative fitness data (v) were first log-transformed to meet the assumptions of parametric analysis. We used a linear model to test whether the relative fitness of non-producers ($\log(v)$) changed significantly according to their initial frequency, iron availability and presence of phage. We estimated fluorescence and bacterial density using optical density readings over the duration of the 12 h growth experiment as area under the curve (MESS package [44]).

3. Results

(a) Growth in monocultures in the absence and presence of phage

Consistent with previous studies (e.g. [39,45]), monocultures of both producers and non-producers grew significantly less under iron-limited than under iron-rich conditions ($F_{1,24} = 77.91$, $p < 0.0001$ at T1 and $F_{1,31} = 1590.87$, $p < 0.0001$ at T6; figure 1). As expected, the presence of phage reduced the fitness of bacterial populations after 24 h ($F_{1,24} = 76.67$, $p < 0.0001$), and this decrease was significantly different depending on iron availability and the bacterial strain (phage × iron × strain interaction, $F_{1,24} = 24.08$, $p < 0.0001$). Specifically, under iron-limited conditions, the non-producers grew to significantly lower densities than the producers, in both the presence and absence of phage (figure 1*a*). In contrast,

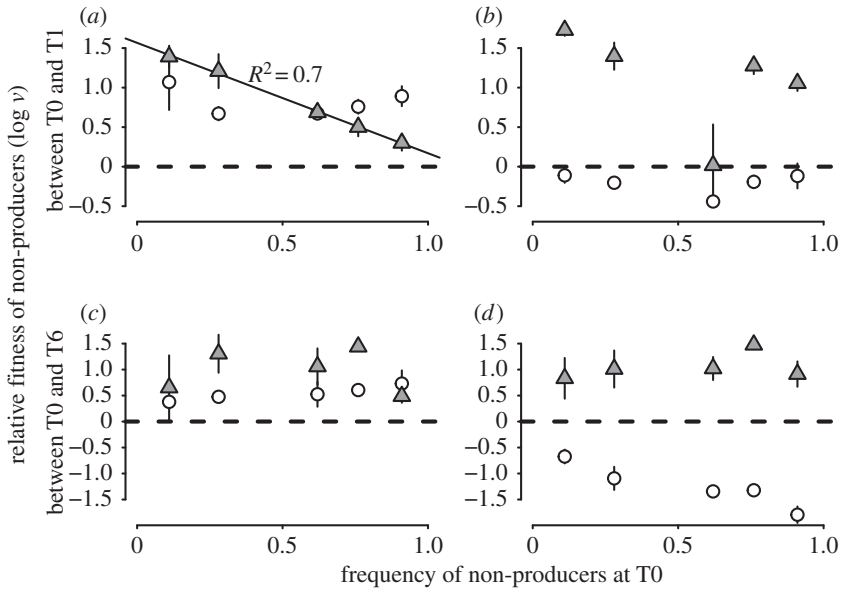

Figure 2. Relative fitness of non-producers (*a*,*b*) between T0 and T1 and (*c*,*d*) between T0 and T6 in (*a*,*c*) iron-limited and (*b*,*d*) iron-rich environments, in the absence (white circles) and presence (grey triangles) of phage. Data are logarithmically transformed (log *v*). Regression is fitted according to the least-squares method. When relative fitness (log *v*) is positive, non-producers increased in frequency, whereas they decreased when negative. The dashed line represents the situation where the frequency of non-producers did not change between two time points. Bars are standard errors of the mean.

under iron-rich conditions, phage did not significantly decrease non-producer growth, but decreased producer growth such that the former grew to significantly higher densities than did the latter (figure 1*b*).

After five serial transfers in the iron-limited environment, producers reached significantly higher densities than non-producers, both in the presence and the absence of phage ($F_{1,15} = 158.95$, $p < 0.0001$; figure 1*c*). Moreover, phage significantly decreased densities of non-producers, whereas it did not affect densities of producers ($F_{1,15} = 83.14$, $p < 0.0001$; figure 1*c*). This finding is supported by a streaking assay that revealed a lower level of resistance in non-producers (17.8% ± 2.8%) than in producers (97.8% ± 1.1%) ($F_{1,216} = 20.5$, $p < 0.0001$). By contrast, in iron-rich environments, there was no significant difference in growth between producers and non-producers ($F_{1,16} = 0.09$, $p = 0.77$; figure 1*d*) and both grew less in the presence than in the absence of phage ($F_{1,16} = 7.18$, $p < 0.05$). Yet they both attained high densities, indicating that these bacteria may have evolved resistance to their sympatric phages. In the streaking assay, we detected high levels of resistance in both producers and non-producers (overall, more than 97% of the tested colonies were resistant to sympatric phage), with resistance slightly higher in producers (99.4 ± 0.55%) than in non-producers (95.5 ± 1.54%) ($F_{1,356} = 5.92$, $p < 0.05$).

(b) Relative fitness in the presence and absence of phage

As expected under iron-limited conditions and in the absence of phage, non-producers grew to higher densities when in competition with producers than they did in monocultures ($t_{14} = 3.95$, $p < 0.01$ at T1 and $t_9 = 5.27$, $p < 0.001$ at T6). In contrast, producers showed reduced growth in competition compared with in monocultures ($t_{27} = -7.23$, $p < 0.0001$ at T1 and $t_{15} = -2.43$, $p < 0.05$ at T6). These results indicate that non-producers benefited from the presence of producers at a cost to the latter at both T1 and T6. Consistent with these findings, non-producers had a competitive advantage over producers when iron was limiting ($t_{24} = 10.22$, $p < 0.0001$ at T1 and $t_{23} = 7.54$, $p < 0.0001$ at T6; electronic supplementary material, figures S1A and 2A). However, non-producers decreased in frequency under iron-rich conditions without phage, at both T1 and T6 ($t_{19} = -4.65$, $p < 0.001$ at T1 and $t_{24} = -14.03$, $p < 0.0001$ at T6; electronic supplementary material, figures S1B and 2B). This result suggests that the pvdD gene may be involved in other fitness-related functions [39].

In the presence of phage in the iron-limited environment, the fitness of non-producers was higher in mixed cultures than in monocultures ($t_{14} = 5.84$, $p < 0.0001$ at T1 and $t_{11} = 3.52$, $p < 0.01$ at T6), whereas producers grew better in monocultures than in mixed cultures ($t_{27} = -4.64$, $p < 0.0001$ at T1 and $t_{25} = -10.99$, $p < 0.0001$ at T6). These results indicate that non-producers were cheating on producers under phage pressure, at both T1 and T6. Iron availability showed a significant interaction with the presence of phage at both T1 and T6, such that the impact of phage on the relative fitness of non-producers was different depending on iron conditions ($F_{1,84} = 50.51$, $p < 0.0001$ at T1 and $F_{1,88} = 69.15$, $p < 0.0001$ at T6; figure 2). Under iron-rich conditions, phage presence reversed the outcome of competition between producers and non-producers, conferring an advantage to the latter, whereas they were outcompeted in the absence of phage ($t_{18} = 6.27$, $p < 0.0001$ at T1 and $t_{23} = 7.41$, $p < 0.0001$ at T6; figure 2*b*,*d*). Under iron-limited conditions, the presence of phage increased the advantage of non-producers at T6 ($t_{31} = -2.94$, $p < 0.01$; figure 2*c*). At T1, we found a significant interaction between the effect of phage and the initial frequency of non-producers ($F_{1,83} = 12.94$, $p < 0.001$; figure 2*a*). Under these conditions, the relative fitness of non-producers was negatively associated with their initial frequency under phage pressure ($F_{1,23} = 55.78$, $p < 0.0001$, $R^2 = 0.70$; figure 2*a*). The same trend indicative of negative frequency dependence was observed in the iron-rich environment in the presence of phage, but was only statistically significant when

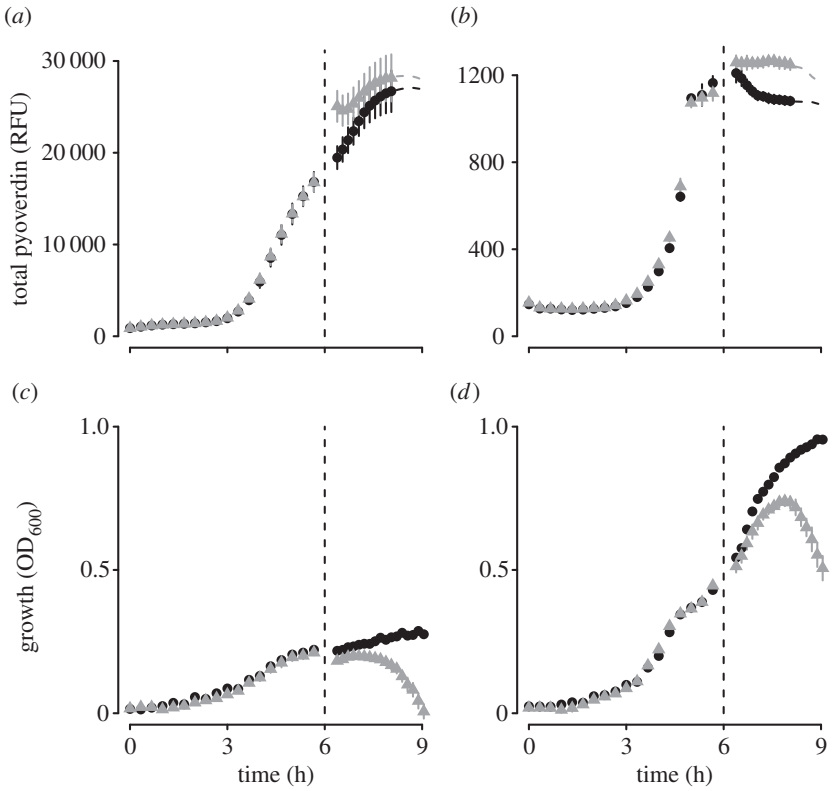

Figure 3. (*a*,*b*) Pyoverdin accumulation and (*c*,*d*) growth curves of producers in (*a*,*c*) iron-limited and (*b*,*d*) iron-rich environments in the absence (black circles) and presence (grey triangles) of phage. Phages were introduced after 6 h (vertical dashed line). Bars are standard deviations of the mean.

a highly variable treatment (with initial frequency of 62% non-producers) was removed ($F_{1,13} = 12.8$; $p < 0.01$, $R^2 = 0.46$; figure 2*b*).

When iron availability was limited, mixed populations of producers and non-producers grew and reached high densities in the presence of phage at T1 (electronic supplementary material, figure S2A). After five transfers, however, these mixed populations were decreasing and only the producer monocultures actually grew under phage pressure ($t_{23} = 4.49$, $p < 0.0001$; electronic supplementary material, figure S2C). Conversely, there was no significant difference in densities at T6 between the mixed populations and the monocultures under iron-rich conditions (contrasts SNK, $p > 0.05$; electronic supplementary material, figure S2D).

(c) Testing hypotheses to explain the differential effect of phage on the producers

We tested two non-mutually exclusive hypotheses to explain our finding of phage having a larger impact on producer compared with non-producer populations. First, we tested whether phages were initially either more infective and/or more productive on producers than on non-producers. In the streaking assay, no colony was scored as resistant for either ancestral bacterial strain against the ancestral phage. Furthermore, in the plaque assay to test phage production, we found no evidence for higher phage fitness in the producers compared with the non-producers ($w_{\text{producers}} = 0.26 \pm 0.03$ s.d. and $w_{\text{non-producers}} = 0.29 \pm 0.02$ s.d.; $t_{10} = 1.9$, $p = 0.09$).

A second hypothesis to explain our finding is that phages imposed an additional cost on producers due to the overproduction of pyoverdin. We measured pyoverdin in the T6 microcosms, where the densities of producers in the presence and absence of phage in monocultures are similar ($t_{17} = 1.33$, $p = 0.2$), since a previous study showed that pyoverdin production is modified in response to cell densities [39]. We found that in monocultures, the amount of pyoverdin is higher in the presence than the absence of phage for both iron environments ($\text{RFU}_{\text{without phage}} = 10\,174 \pm 820$ s.d., $\text{RFU}_{\text{with phage}} = 12\,099.4 \pm 298$ s.d., $t_5 = -4.93$, $p < 0.01$, $n = 10$ in iron-limited environment and $\text{RFU}_{\text{without phage}} = 869 \pm 125$ s.d., $\text{RFU}_{\text{with phage}} = 1210 \pm 91$ s.d., $t_7 = -4.71$, $n = 10$, $p < 0.01$ in iron-rich environment).

We also tested the second hypothesis by following the density and pyoverdin production of ancestor producer monocultures. In support of this hypothesis, we observed higher fluorescence and therefore pyoverdin quantity in the populations with phage than without phage, under both iron conditions ($t_8 = -4.65$, $p < 0.005$ under iron-limited conditions and $t_8 = -7.42$, $p < 0.0001$ under iron-rich conditions; figure 3).

4. Discussion

In this study, we investigated the impact of phage predation in both ecological and evolutionary time on bacterial public goods production strategies. We observed that the presence of phage increased the frequency of non-producing mutants, both when the production of siderophores is and is not beneficial for bacterial growth. Specifically, non-producers increased more in frequency in the presence than in the absence of phage in the iron-limited environment. Under iron-rich conditions, whereas the producers were at an advantage without phage, non-producers outcompeted them in the presence of phage. In the iron-limited environment with phage, the advantage to non-producers in mixed cultures ultimately led to decreased population densities, arguably due to the lack of siderophores to sustain growth. These results highlight the

importance of considering interspecific interactions under different environmental conditions when studying the evolution of public goods strategies and, more generally, social evolution and intraspecific cooperation and competition.

Previous studies have investigated how natural enemies may affect the outcome of public goods production in microbial systems. For example, predators have been shown to impact the interactions in *Pseudomonas* species in favour of producers due to the differential killing of non-producers [19,20]. In contrast, Morgan *et al*. [17] reported that phage promotes increases in the most frequent type regardless of its production strategy, because that type would be more likely to be associated with phage-resistant mutants. We found that phage always selected for an increase in non-producers and found no evidence for a differential effect of phage on ancestral producers and non-producers in monocultures. Moreover, the relative fitness of non-producers in the presence of phage was significantly negative frequency dependent during the first 24 h, consistent with phage selecting against the more frequent siderophore production strategy. We therefore suggest that the overall impact of phage on temporal changes in the two bacterial types is influenced not only by initial conditions but also by subsequent complex ecological and evolutionary dynamics. The seemingly contradictory outcomes of our work and the study by Morgan *et al*. [17] may be explained by the use of a different phage–bacteria system, the employment of different initial phage and bacteria densities, growth media and volume, and/or the employment of different ranges of initial non-producer frequencies. For Morgan *et al*., non-producers decreased in frequency when initially at low frequencies ($\leq$1%), whereas the lowest initial frequency of non-producers in our protocol was 11%.

In our experiment, both the producers and the non-producers have functional siderophore receptors. Therefore, even if phage were able to exploit these receptors as alternative binding sites, as observed in long-tailed phages of *Escherichia coli* [46], this could not explain the higher effect of phage on producer densities. Moreover, we suggest that this phenomenon is unlikely in our system since the short-tailed phage LKD16 employs type IV pili as a receptor [47] and the fluctuation selection dynamics it undergoes with bacteria is indicative of high specificity to this binding site [48].

Our results suggest that the dynamics of competition and producing strategies are mediated by siderophore availability. Higher overall production of pyoverdin leads to selection for non-producers by increasing the cost-to-benefit ratio for production. Harrison *et al*. [49] reported that the presence of an interspecific competitor led to competition for iron. This in turn resulted in higher siderophore production, thus increasing the advantage to defecting mutants. Our results indicate that the presence of phage favoured non-producers in mixed cultures not only under iron-limited, but also under iron-rich conditions, due to an increase in pyoverdin production. This can be understood as follows. When iron availability is not limiting, there is little or no benefit to pyoverdin production for either producers or non-producers. However, because non-producers do not pay production costs, they would have a net advantage over producers, and this may explain why the former outcompeted the latter in mixed cultures, and why producer recovery in monocultures is much slower than that of non-producers. As siderophores are not essential to import iron from the environment in our iron-rich experimental treatments, non-producers can grow in the absence of producers and all populations reached similarly high densities after five transfers. In contrast, when iron availability is limiting, pyoverdin production comes at a cost, but results in a net benefit to producers as it increases access to iron (e.g. [38,45]). Indeed, we found that producers evolved higher levels of resistance to phage in monocultures than did non-producers, suggesting that pyoverdin led to higher growth rates and increased the probability of resistance evolution to the phage. In mixed cultures, non-producers rely on siderophores from producers and the former are selected because they benefit without paying a cost. Their advantage, however, is negatively correlated with their initial frequency: when initially rare, the high pyoverdin-to-mutant ratio allowed mutants to increase rapidly to high frequencies as iron became more available, whereas when initially abundant, the amount of pyoverdin (and thus of available iron) per non-producer cell was lower and their fitness advantage was thereby decreased. Furthermore, while the advantage to non-producers in mixed cultures resulted in high population productivity in the presence of phage at T1, the combined phage and cheating load was associated with decreases in density in these mixed populations by the end of the experiment. Indeed, the decrease in producer frequency and the dilution effect linked to serial transfers both probably contributed to reduction in siderophore concentration resulting in insufficient iron availability to sustain population growth.

Moreover, previous studies together with our results suggest that the cost to producers in the presence of phage may be higher than that due to pyoverdin production alone, since phage may affect other traits such as motility, biofilm formation and pyocyanin production [50], and because pyoverdin is also a signalling molecule that may upregulate other costly behaviours (e.g. the production of exotoxins and endoproteases [51], and biofilms [52]). Further research is needed to elucidate the complex interrelationships between putative social and other life-history traits.

Although we did not investigate the mechanism by which phage increased pyoverdin production, we propose three non-mutually exclusive hypotheses for upregulation. First, upregulation may be a form of 'terminal investment' [31], whereby bacteria increase their survival chances in a stressful, uncertain environment. An increase in siderophore production may have both direct and indirect effects: it can favour growth through higher access to iron and/or regulate other potentially beneficial traits such as biofilm formation (e.g. [53]). Second, quorum sensing has been shown to influence phage–bacteria interactions [54], and an increased activation of this system in the presence of phage may lead to the upregulation of public goods production and of pyoverdin in particular (e.g. [55]; but see [56]). Third, upregulation may be a consequence of phage manipulating bacteria to increase the former's own fitness, and there is some evidence that phage can impact host biology and behaviour (e.g. [57,58]). Given that biological systems and natural environments of both bacteria and phage are generally poor in free ferric iron [59], high local concentrations of siderophores would favour both bacterial growth and the fitness of phage progeny. Future study should test these and other hypotheses for siderophore upregulation (see also [31,34] for associations between phage and increased bacterial growth).

Finally, although we focused on proximal bacterial siderophore production, our results have implications for the evolution of public goods cooperation. We found that in iron-limited monocultures, producer bacteria were able to recover from phage predation, suggestive of resistance evolution [48],

whereas non-producers did not show this effect. In contrast, in iron-limited mixed cultures (which are the relevant situations for social evolution), phages initially selected for increasing frequencies of non-producers, apparently because producers were not able to evolve resistance. Inability to evolve resistance is likely to have been due to producers being rapidly reduced in numbers (and therefore evolutionary potential) by non-producers. These findings indicate that the phage selected against the producer strain and this, together with the relative advantage that non-producers had under iron-limiting conditions, resulted in higher non-producer frequencies in the 'social' environment. However, this advantage led to decreased population productivity, arguably due to the combined phage and cheating load, consistent with our finding that only producer monocultures were able to cope with phage pressure under iron-limited conditions. Future studies should investigate the impact of phage predation on social evolution in spatially explicit systems, where our results would predict that the order of arrival of each population in a given patch will influence the social evolutionary dynamics in that patch, and at larger spatial scales, relative migration rates should have important effects on overall levels of cooperation and its spatial variation [60].

5. Medical implications

Beyond the fundamental findings that phages influence public goods production, our work has implications for the possible medical use of phage. *Pseudomonas aeruginosa* is a multi-drug-resistant bacterium responsible for many nosocomial infections, and particularly complications in cystic fibrosis patients. Combined therapies using both antibiotics and phage, although mostly tested *in vitro* (e.g. [61]), have considerable potential in controlling certain human bacterial infections [62]. Understanding the effect of phage on pyoverdin dynamics is of particular interest given the role of siderophores in bacterial virulence [38]. Our results show that phage can select for non-siderophore-producing bacterial variants, suggesting that colony virulence should be lessened compared with colonies dominated by producers [38]. This advantage to non-producers was observed under two medically relevant iron conditions: an iron-limited environment wherein hosts scavenge and retain iron using iron-binding proteins as an innate immune response to bacterial infections [63], and an iron-rich environment, as observed in chronically infected tissues [64]. However, we also showed that phage upregulate siderophore production, and therefore suggest that in viscous medium where siderophores do not readily diffuse (as would be expected in many *in vivo* situations), this would select for producers and lead to higher virulence [38]. These points highlight the importance of understanding the complex interplay between public goods dynamics and phage predation in the design of phage therapies.

Authors' contributions. M.V., C.T.-B. and M.E.H. conceived and designed the experiments. M.V. performed the experiments and analysed the data. M.V. and M.E.H. wrote the manuscript and all authors contributed substantially to revisions. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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