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Research Article

Evolution of exploitation and replication of giant viruses and virophages

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Abstract

Tripartite biotic interactions are inherently complex, and the strong interdependence of species and often one-sided exploitation can make these systems vulnerable to extinction. The persistence of species depends then on the balance between exploitation and avoidance of exploitation beyond the point where sustainable resource use is no longer possible. We used this general prediction to test the potential role of trait evolution for persistence in a tripartite microbial system consisting of a marine heterotrophic flagellate preyed upon by a giant virus, which in turn is parasitized by a virophage. Host and virophage may benefit from this interaction because the virophage reduces the harmful effects of the giant virus on the host population and the virophage can persist integrated into the host genome when giant viruses are scarce. We grew hosts and virus in the presence and absence of the virophage over ∼280 host generations and tested whether levels of exploitation and replication in the giant virus and/or virophage population evolved over the course of the experiment, and whether the changes were such that they could avoid overexploitation and extinction. We found that the giant virus evolved toward lower levels of replication and the virophage evolved toward increased replication but decreased exploitation of the giant virus. These changes reduced overall host exploitation by the virus and virus exploitation by the virophage and are predicted to facilitate persistence.

Keywords: giant virus; *cafeteria burkardae*; virophage; evolution; persistence.

Introduction

Virophages are a distinct class (*Maveriviricetes*) of viral agents that parasitize the cytoplasmic factories of giant viruses in the order *Imitervirales* for their replication (Duponchel, Fischer, and Condit 2019; Roux et al. 2023). Metagenome studies found that virophages can be highly abundant and diverse, which suggests that they play an important role in the dynamics of microbial communities by modulating giant virus-mediated mortality in protist populations (Paez-Espino et al. 2019). The interaction between hosts and virophages has been described as being mutualistic because virophages facilitate host persistence in the presence of high giant virus densities, and because host cells provide an opportunity for the virophage to persist integrated into the genome of the host when giant viruses are scarce (Fischer and Hackl 2016; Koonin and Krupovic 2016; Berjón-Otero, Koslová, and Fischer 2019; Hackl et al. 2021). The benefit for the host arises because virophages reduce the lytic impact of the giant virus on the host population over time by inhibiting giant virus replication. However, there are less data on long-term two-way interactions between host and virophage at the population level and giant virus and virophage trait evolution. Like other tripartite systems with strong interdependence of interacting species and high levels of exploitation, i.e. the benefits of the interaction are one-sided (e.g. hyperparasitism, satellite viruses, and helper viruses (May and Anderson 1983; Ewald and Ewald 1987)), the host–virus–virophage systems may be short-lived and prone to extinction (Holt and Hochberg 1998; Wodarz 2013).

The long-term persistence of host, giant virus, and virophage largely not only depends on the strength of virophage–giant virus and giant virus–host parasitism but may also depend on environmental factors (e.g. predators of the host, seasonal population density changes) (Yau et al. 2011; Biggs, Huisman, and Brussaard 2021). Overexploitation by the giant virus can lead to extinction of the host (followed by the virophage and the giant virus, unless they expand their host range), while overexploitation by the virophage can lead to extinction of the giant virus (cf., the Tragedy of the Commons). The latter would also reduce the possibility for horizontal transfer of the virophage through infection of new hosts with potential long-term consequences for virophage fitness. The level of exploitation depends on the traits of the consumer (giant virus and virophage), resource (host and giant virus), and population sizes. For long-term stability of the system, traits are predicted to evolve in a way that adaptations allow for sufficient exploitation without compromising the abundance of the host as

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a resource (Morozov, Robin, and Franc 2007; Heilmann, Sneppen, and Krishna 2010; Wodarz 2013). This balance may be difficult to evolve for the giant virus because different traits might determine the level of host exploitation and exploitation by the virophage, and negative associations between traits may constrain trait evolution. This has been shown for hyperparasitism (May and Hassell 1981; Addy et al. 2012; Parratt and Laine 2016), when hosts evolve in the presence of a virus/phage and predator (Gómez, Buckling, and Grover 2013; Frickel, Theodosiou, and Becks 2017) or when hosts evolve in the presence of defensive and pathogenetic bacteria (Ford et al. 2016). For example, higher virulence of a pathogen and thus higher exploitation can evolve when virulence and the pathogen's resistance against a competitor or a defensive microbe (e.g. virophage) are positively correlated (Santander and Robeson 2007; León and Bastías 2015). Lower virulence against the host was found when virulence is costly with respect to resistance against the defensive microbe (León and Bastías 2015; Ford et al. 2016). Traits contributing to exploitation and replication, their evolution, and potential roles for the persistence of host–virus–virophage systems are, however, largely unexplored.

Here, we use experimental evolution to study virus and virophage traits and how their evolution might contribute to the long-term persistence of host–virus–virophage systems. We established the marine bacterivore *Cafeteria burkhardae*, the giant virus *Cafeteria roenbergensis* virus (CroV), and the virophage mavirus (Fenchel and Patterson 1988; Fischer et al. 2010; Fischer and Suttle 2011; Schoenle 2020) in chemostats (del Arco, Woltermann, and Becks 2020). Hereafter, we refer to CroV as 'virus' and to mavirus as 'virophage'. We studied virus and virophage trait evolution by comparing their replication and exploitation between ancestors that were used to start the chemostats and re-isolated virus and virophage from day 57 (after ∼280 host generations). In this system, virus infection of the host nearly always leads to cell lysis. During a virophage-only infection of the host, the virophage may integrate into the nuclear genome and reproduce as a provirophage in the host genome (Fischer and Hackl 2016). When virus and virophage coinfect a host cell, virophage production blocks virus capsid formation and only virophages are produced, which mitigates the effect of the virus on the host population during subsequent infections. When the virus infects a host with integrated virophage in their genome, both virus and virophage are produced, with no apparent decrease in virus replication compared to virus-only infections. Here, we tested the specific prediction that trait evolution leading to lower virophage replication could contribute to host–virus–virophage persistence (Wodarz 2013). This can result from low intrinsic growth and/or high pathogenicity of the virophage towards the virus. Specifically, a higher degree of exploitation of the virus over time results in lower virus densities and thus fewer opportunities for the virophage to parasitize it and increase its exploitation. In addition, we tested whether the virus evolved a higher reproductive output in the absence of virophage and a lower reproductive output in the presence of the virophage (Wodarz 2013), resulting in higher and lower host exploitation, respectively. Lower levels of virus replication reduce exploitation by the virophage and thus its replication and population size over time.

Materials and methods

Virus and virophage evolution experiment

Our experimental system consisted of the marine heterotrophic flagellate *C. burkhardae* strain E4-10P as host, the giant virus CroV, and its virophage mavirus. The experiment was performed in continuous chemostat systems (glass bottles with 400 ml f/2-enriched artificial seawater (SW) medium (Guillard and Ryther 1962) supplemented with 0.025 per cent (w/v) yeast extract and 0.3 g/ml of chloramphenicol) with a continuous flow-through of 120 ml medium per day (= 0.3× dilution rate per day) (del Arco, Woltermann, and Becks 2020) at 18 ± 0.5°C. The chemostat experiments lasted for 57 days, based on host density data collected immediately after daily sampling, which indicated that host densities became very low in the virus treatments (Supplementary Fig. S1). The 57 days represent up to 280 host generations (number of generations under optimal replication conditions of the host in control groups assuming a doubling time of 4.2–4.6 h [Massana et al. 2007]). Triplicate chemostats were inoculated with the host and *Escherichia coli* bacteria (control), with *E. coli* and the virus (virus treatment), and with *E. coli*, virus, and virophage (virus–virophage treatment). All chemostats were inoculated with $7{*}10^4$ host cells/ml, and both virus and virophage were added at day 7 and again at day 13 when host population dynamics had reached maximum population sizes. Viruses and virophage were inoculated at a virus-to-host ratio of 0.1 in the virus and virus–virophage treatments based on host density counts and virus and virophage DNA copies quantification (del Arco, Fischer, and Becks 2022). A virus–host ratio of 0.1 would allow initial virus and virophage replication without driving the host to extinction (Fischer and Hackl 2016). As these chemostat experiments ran for 57 days, ratios changed because of ecological and evolutionary dynamics. We used a chloramphenicol-resistant *E. coli* strain (BL21(DE3)pLysS) to keep the bacterial community composition constant. *Escherichia coli* served as food for the host in our experiments. The flagellate host strain used to start the experiments carried endogenous marvirus (Hackl et al. 2021), but these did not produce mavirus particles under the experimental conditions of this study as has not been observed in similar experimental conditions (Supplementary Information, Fig. S3). Therefore, we consider the control and the virus treatment to be lacking free marvirus virophages which we confirmed by the absence of amplification of free virophage from the control and virus treatment at day 57 of the experiment (see below for virus quantification; for a discussion of the potential role of other endogenous virophage and MAvirus-Like-Elements (EMALE, Hackl et al. 2021), see 'Discussion' section).

We sampled chemostats at the end of the experiment on day 57 to determine host densities and the presence of virus and virophages. The host was quantified from live samples using a hemacytometer and light microscope (20× magnification). For the virus and virophage, we separated viruses from host cells, bacteria, and from each other by filtration through 0.45 μm and 0.2 μm filters and quantified in each fraction the DNA copies/ml by digital droplet PCR (ddPCR). For all ddPCR assays, DNA was extracted using a commercial kit (DNeasy 96 Blood & Tissue Kit, Qiagen, Hilden, Germany) and kept at 4°C until the ddPCR. We designed and selected primer and probes for virus amplification as described earlier (del Arco, Fischer, and Becks 2022). PCR parameters were set to one cycle of 95°C (10 min), forty cycles of 94°C (30s), forty cycles of 58°C (1 min), one cycle of 98°C (10 min), and a hold temperature of 12°C. All ddPCR results were analyzed using QUANTASOFT 1.7.4. The detailed methods and quality requirements for the data are described in the reference (del Arco, Fischer, and Becks 2022). Viruses and virophages differ in capsid sizes: 300 nm for CroV and ∼70 nm for mavirus (Fischer and Suttle 2011; Xiao et al. 2017). We used a Spartan® cellulose filter (Whatman®) with a pore size of 0.2 μm to separate viruses and virophages from mixed samples. With a filter pore size of 0.2, CroV is mostly retained on the

filter, while mavirus passes through the filter. We used reverse filtration to recover CroV from the filter (see further). Retention of CroV on the filter with this protocol was high, resulting in too few virus particles for further experiments; hence, we amplified these viruses by another round of infection before using them in a series of assays to detect phenotypic changes that developed during the experiment. We refer to *ancestral* virus and virophage lines for the virus and virophage that were used to inoculate the chemostats and to *selected* virus and virophage lines for the viruses and virophages that were isolated and amplified at the end of the experiment. We used the selected viruses versus ancestral populations to measure levels of exploitation and replication under standardized conditions (see further). Differences between selected and ancestral population DNA copy numbers allowed us to identify heritable phenotypic changes in the virus populations following standard procedures of experimental evolution (Gómez, Buckling, and Grover 2013; Frickel, Sieber, and Becks 2016).

We did not follow virus and virophage densities over time and thus we have no information on virus and virophage replication. It is theoretically possible, but highly unlikely given the continuous dilution of chemostat cultures (dilution rate = 0.3/day), that the selected virus and virophage, which were amplified from the end of the experiment, are residual ancestral virus and virophage or were produced at the beginning of the experiment rather than virus and virophage produced throughout the experiment. The selection of virus and virophage over the experiment is supported by the observation that virus and virophage replication and exploitation differed when we compared ancestral and selected populations in standardized assays.

Virus and virophage amplification

For virus amplification, we added 50 µl of the medium used to recover viruses from the 0.2 µm filter to the medium containing the ancestral host (strain E4-10P; 105 cells/ml) to propagate and produce virus stocks of isolated virus populations from day 57 of the chemostat experiment. Virus amplification was done for each chemostat replicate of the virus and virus–virophage treatments (one replicate was lost from the virus treatment, see also Supplementary Fig. S2). After observing host lysis (confirmed by sampling and enumeration by microscopy), we collected virus samples (filtration through 0.45 µm filters) and quantified virus DNA copies/ml by ddPCR (as mentioned earlier). To produce selected virophages, we added 50 µl of the filtrate of the 0.2 µm filter to medium containing strain host (strain E4-10P; 10^5 cells/ml) and ancestral virus. After observing host lysis, we collected virophage samples (filtration through 0.2 µm filter) and quantified virophage DNA copies/ml by ddPCR.

Recovery of the virus fraction

We used reverse filtration to recover the virus fraction retained in the 0.2 µm filter (see earlier for separation of size fractions). For this, we attached a flexible tube (6 mm diameter) to the back of the filter and washed it with the medium used in the experiment to recover CroV. We measured the number of virophage DNA copies in the virus fraction (CroV) that were not removed from the virus fraction using this approach. On average, we detected $\sim 10^3$ virophage DNA copies/ml, resulting in a virophage–host ratio of ~0.2 when considering the sample volumes added to achieve a virus–host ratio of 0.1 in the assays described earlier. In these assays, we detected amplification of virophages in three of nine samples (chemostat C: 1 out of 3; chemostat D: 1 out of 3; chemostat E: 0 out of 3; for virus amplification and assay description see further). But there were no differences in the densities of amplified viruses when comparing the densities of replicates with and without virophage amplification from the same chemostat.

Assay for virus and virophage evolutionary changes

We tested whether viruses and virophages evolved during the chemostat experiment by comparing the replication and exploitation of ancestral and selected viruses and virophages. We used host and virus densities as proxies for the degree of virus and virophage exploitation and replication. We infected the ancestral host (strain E4-10P) in the 27 ml SW medium in tissue culture flasks with ancestral or selected viruses at a virus–host ratio of 0 (virus-free control) or 0.1 and with ancestral or selected virophages at a virus–host ratio of 0 (no virophage added control) or 10. This ratio allows the detection of produced virus particles after 1 day of infection (Fischer and Hackl 2016). Note that the background virophage in the virus fraction after reverse filtration (as mentioned earlier) leads to a low virophage-to-host ratio of 0.2 in the treatments where the virus came from the virus–virophage treatments. This had a negligible effect on the assays where we started with a ratio of 10 but could have had effects on virus replication in the assays where we did not add virophage, and the selected virus was amplified from the virus–virophage treatments. We discuss the potential effect of this background virophage below (see 'Discussion' section). Therefore, we assume that the effect of the background virophage in the selected virus from the virus-virophage treatment had no detectable effect on the virus replication measurements.

All conditions were performed in triplicates, starting with a host density of 10^4 cells/ml. Host and virus samples were collected and quantified 1 day after infection. Host samples were preserved with glutaraldehyde (4 per cent final concentration). Hosts were quantified by flow cytometry (FACSverse). For this purpose, samples were stained with SYBR-Green I DNA stain (Sigma-Aldrich) at a final concentration of 0.02 per cent. Host enumeration was performed in well plates at a flow uptake of ~90 µl for 24 s with shaking at 1,000 rpm. Lateral light scatter (SSC) and SYBR-green fluorescence (FITC) measurements were performed with logarithmic amplification using FlowJo software (BDBiosciences). Virus and virophage samples were immediately collected for DNA extraction and quantified by ddPCR (see above and (Del Arco, Fischer, and Becks 2022)).

Free and integrated virophage in host genome

We tested whether the number of virophages integrated into the genome and the presence of free virophages in the absence of the virus affected host growth using four different host strains: *C. burkhardae* strain E4-10P; strain RCC970-E3 and two strains derived from the strain RCC970-E3: strain RCC970-E3-8.8 and strain RCC970-E3-9.3.1 with two or three copies of integrated virophage genomes, respectively. Host growth was tested in the presence and absence of free virophages (virophage–host ratio of 8) in tissue culture flasks containing a 30 ml SW medium and in five replicates (host: $2*10^3$ cell/ml). Samples were collected 6 days after infection, fixed (4 per cent final concentration of glutaraldehyde), and host densities quantified by flow cytometry (see above).

Data analysis

All data analyses were performed in Rstudio (RStudio Team, V. 2020) and R (R Core Team, V. 4. 1. 1. 2020) using the packages geepack (Højsgaard et al. 2020) and lme4 (Pinheiro et al. 2017).

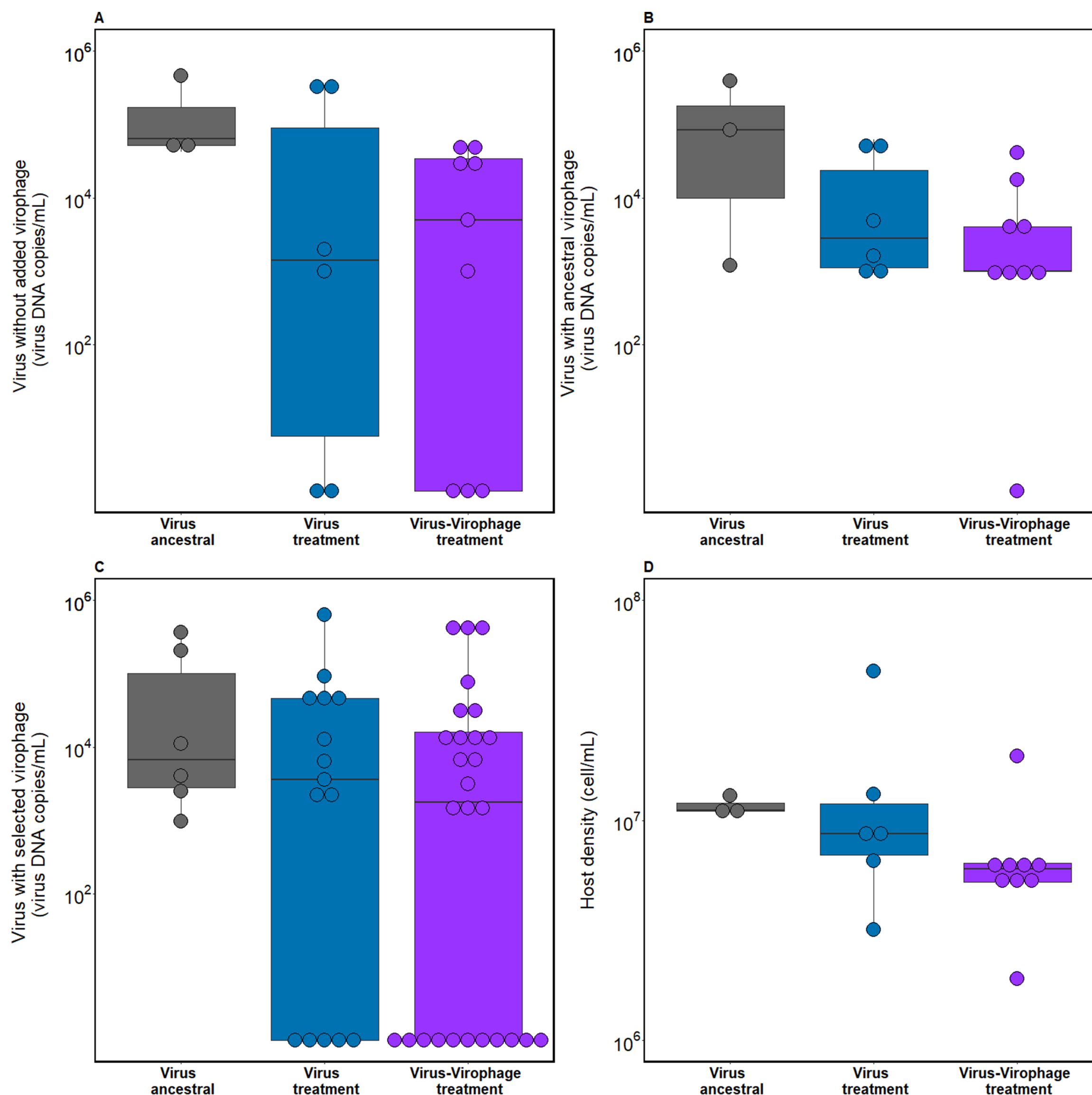

Figure 1. Changes in virus replication and host exploitation. Densities of ancestral and selected virus lines (as viral DNA copy numbers/milliliter, median). (A) Virus densities without virophages, (B) virus densities with ancestral virophages, (C) virus densities with selected virophages, and (D) host densities with ancestral virus or selected lines but without added virophage. Gray = ancestral, blue = selected virus from chemostat with virus, purple = selected virus from chemostat with virus and virophage. There were three replicates per virus type (either ancestral or selected): one ancestral virus, two selected viruses from two chemostats of the virus treatment, three selected viruses from three chemostats of the virus–virophage treatment and three selected virophages from the three chemostats of the virus–virophage treatments (Supplementary Fig. S2). Depending on the host–virus combinations, the number of data points varies between panels because each virus or virophage isolate was challenged with viruses from the same and different chemostats (Supplementary Fig. S2).

Significance was evaluated using model comparisons. Differences between statistical models were considered relevant when $P < 0.05$. All posthoc tests included corrections for multiple testing (Tuckey) using the package (multcomp) (Hothorn, Bretz, and Westfall 2008). We used the generalized linear mixed model (GLMER, family = poisson) to test for differences in virus densities to evaluate differences in virus replication and exploitation (Fig. 1A–C). For these tests, we used virus (levels: ancestral, selected from the virus treatment, selected from the virus–virophage treatment) and virophage (levels: absent, ancestral, selected) as well as their interaction as explanatory variables and chemostat as random effect to account for the relatedness of selected virus and virophage isolated from the same chemostat. To compare host densities in the presence of the virus, we used a GLMER with virus (levels: ancestral, selected from virus treatment, selected from virus–virophage treatment) as explanatory and chemostat as random variable (Fig. 1D). We used a GLMER (family = poisson) to test for differences in virophage DNA copy numbers with virophage (ancestral, selected), virus (levels: ancestral, selected from the virus treatment, selected from the virus–virophage treatment), and their interaction as explanatory and chemostat as random variable (Fig. 2). We compared host densities in the presence of virus and presence and absence of virophage (Fig. 3) using a GLMER (family = poisson) with virus (levels: ancestral, selected from the virus treatment, selected from the virus–virophage treatment), virophage (ancestral, selected), and their interac-

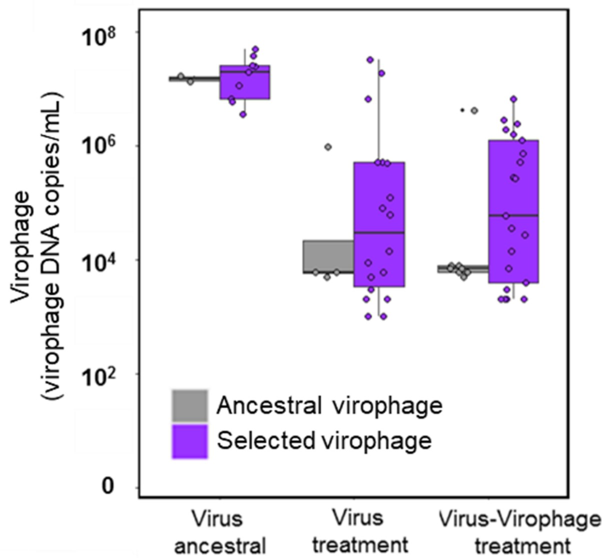

Figure 2. Changes in virophage replication. Virophage DNA copy numbers (DNA copy numbers/milliliter, median) of ancestral (gray) and selected virophage lines (purple) in the presence of ancestral virus 1 day postinfection.

tion as explanatory variable. For all tests, we used generalized linear models as we were dealing with count data including zero values.

Results

Virus and virophage evolution experiment

We measured host population and tested for the presence of virus and virophage at the end of the experiment and confirmed the presence of host, virus, and virophage. We found that the host–virus–virophage could persist over the course of the experiment without driving each other to extinction. Host reached densities of $8*10^5$ host cells/ml in average, and virus and virophage were amplified for further experiments.

Virus evolution: decreased replication

We compared virus densities of ancestral and selected virus (virus and virus–virophage treatment) in the presence or absence of virophage (ancestral or selected) 1-day postinfection. We found that ancestral and selected virus densities differed significantly and depended on whether ancestral or selected virophage was present (Fig. 1, GLMER: virus χ^2= 366,324, df = 6, $P < 0.001$; virophage χ^2= 7,760,494, df = 6, $P < 0.001$; interaction virus and virophage χ^2= 366,321, df = 4, $P < 0.001$). Specifically, we found that virus DNA copy numbers were lower for selected virus from the virus–virophage treatment compared to the ancestral virus with and without the virophage (Fig. 1A, posthoc test: $P < 0.0001$; all other comparisons non-significant).

In the presence of ancestral virophages, the DNA copy numbers of selected viruses from both treatments were significantly lower than the DNA copy numbers of ancestral viruses (Fig. 1B, GLMER: virus χ^2 = 8.0843, df = 2, $P = 0.017$). This was not the case in the presence of selected virophages (Fig. 1C, GLMER: virus χ^2 = 3.8981, df = 2, $P = 0.142$). To further assess host exploitation by the virus, we compared host densities 1-day postinfection in the presence of ancestral and selected virus (virus and virus–virophage treatment). Host densities were not significantly different depending on the virus (Fig. 1D, GLMER: χ^2 = 3.94, df = 2, $P = 0.139$).

Virophage evolution: increased replication and decreased virus exploitation

Ancestral virophage DNA copy numbers were significantly higher in the presence of the ancestral virus compared to the selected virus, and virophage DNA copy numbers were higher for selected virophage than for ancestral virophages when tested with ancestral and selected virus (Fig. 2, GLMER: virus line, χ^2 = 10,949,655, df = 4, $P < 0.001$; virophage line, χ^2 = 42,586,791, df = 3, $P < 0.001$; interaction virus and virophage line, χ^2= 10,949,652, df = 2, $P < 2.2*10^{16}$).

Effects of evolution on host: reduced exploitation

To further assess how host exploitation by the virus is affected by the virophage, we compared host densities in the presence of ancestral and selected virus (virus and virus–virophage treatment) and presence of ancestral or selected virophage. Host density was significantly affected by the combination of virus and the virophage lines (Fig. 3, GLMER: virus line, χ^2 = 18,317,684, df = 4, $P < 0.001$; virophage line, χ^2= 42,042,860, df = 3, $P < 0.001$; interaction virus and virophage line, χ^2 = 18,317,680, df = 2, $P < 2.2*10^{-16}$). Specifically, host densities with selected virus from the virus–virophage treatment had significantly higher densities compared to the other two virus lines in the presence of the ancestral virophage (Fig. 3, Posthoc test: ancestral virus versus selected in presence of virus and virophage: $P < 0.0001$; selected in presence of virus *vs.* selected in presence of virus and virophage: $P < 0.0001$; ancestral virus *vs.* selected in presence of virus: $P = 0.978$) and host densities were generally higher in the presence of the selected virophage compared to the ancestral virophage (Fig. 3B, Posthoc test: ancestral virus versus selected virus from the virus and virus–virophage treatment: $P < 0.001$; selected virus from the virus treatment versus selected virus from the virus–virophage treatment: $P < 0.001$; ancestral virus versus selected virus from the virus treatment: $P < 2.2*10^{-16}$).

We assessed if the number of virophages integrated in the genome and the presence of free virophages affected host growth in the absence of the virus by using host strains with different numbers of virophage copies integrated in their genome. We found no effect of integrated or free virophage on the host strain growth rate (Supplementary information Fig. S4, LME, interaction host line and virophage type, χ^2 = −0.73, df = 3, $P = 0.975$).

Discussion

Long-term persistence of consumer and resource (i.e. host–parasite, predator–prey) is only possible if the consumer does not exploit the resource beyond the point where sustainable exploitation is no longer possible. This problem appears to be prominent in systems where one species exploits a resource while being exploited by another species, since adaptation must favor traits that balance exploitation with avoidance of being exploited so that organisms are not driven to local extinction. Here, we explored evolutionary changes in exploitation and replication of a giant virus and virophage as a first experimental assay in host–virus–virophage systems to test the potential role of evolution to persistence of host–virus–virophage systems. By comparing traits between the ancestral and the selected isolates, we found that the virus evolved towards lower replication while host exploitation did not change (Fig. 1A). As we found no differences in virus replication and host exploitation between the virus and virus–virophage treatments (Fig. 1D), we consider the effect of the background virophage to be negligible in these assays. The virophage evolved towards increased replication (Fig. 2) and lower exploitation of the

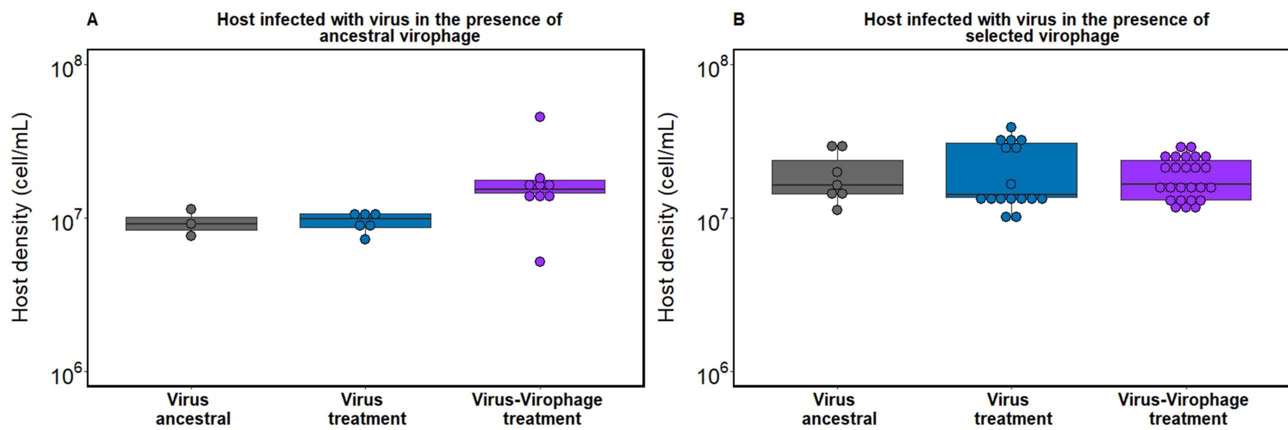

Figure 3. Effect of virophage on host exploitation by the virus. Host density (as cell/ml, median; ancestral host) when grown for 1 day in the presence of virus (ancestral: gray; selected from virus treatment: blue; virus–virophage treatment: purple) and virophage (A: ancestral virophage; B: selected virophage). Host densities were significantly higher in the presence of virus from the virus–virophage treatment with ancestral virophage (A) and in the presence of the selected virophage (B).

virus compared to ancestral virophage (Fig. 1B, C). Lower virus replication as well as lower virus inhibition by the virophage can promote community persistence as these traits reduce the DNA copy numbers of the virus in the community (Fig. 1) and reduce the impact of the virophage on the virus population (Fig. 3). These results are consistent with predictions from mathematical models and show that traits have evolved to allow long-term persistence, e.g. by reducing the probability of virophage extinction as a result of oscillatory dynamics associated with high viral inhibition (Wodarz 2013).

In addition to the evolution of traits that reduce overall exploitation, coevolution between antagonists may facilitate their coexistence (Gómez and Buckling 2011; Hampton, Watson, and Fineran 2020; Piel et al. 2022). For example, the likelihood of prey species coexisting was increased by trait variation resulting from past coevolution between predator and prey (Scheuerl et al. 2020). When selection acts to reduce fitness inequality between competitors, coevolution can promote the long-term coexistence of two competing species (Huang et al. 2017). We also found evidence for virus–virophage coevolution in our experiment: virus replication decreased most in the presence of selected virophages for virus from the virus-virophage treatment, and host exploitation was lowest when virus and virophages from the virus–virophage treatment were combined. Additional experimental tests are needed to further explore this observation, and their results could contribute to a better understanding of diversity in virus–virophage systems. For example, comparing the replication and exploitation of virus and virophage when paired from different time points in the experiment (i.e. time-shift experiments, Gaba and Ebert 2009) could test for the underlying coevolutionary pattern. Knowing whether virus–virophage coevolution is better explained by coevolutionary models with directional selection (arms race dynamics (Frickel, Sieber, and Becks 2016; Frickel, Theodosiou, and Becks 2017)) or by fluctuating polymorphism of virus and virophage (trench warfare dynamics, Stahl et al. 1999) would allow further predictions of the genetic and phenotypic diversity expected in such a system.

The evolutionary changes in virus and virophage observed here affect host and virus exploitation. In general, reduced exploitation can stabilize consumer-resource dynamics (i.e. predator–prey and host–parasite (Rosenzweig and MacArthur 1963), and previous studies have shown that evolutionary changes in traits related to exploitation can affect population dynamics. For example, the evolution of resistance in an algal host that reduced exploitation by a virus resulted in a shift to more stable dynamics with reduced oscillations in population size (Frickel, Sieber, and Becks 2016). Differences in antagonist population size can have a direct effect on the strength of selection, e.g. by reducing the host–virus ratio, and thus on further selection. This link between host–virus ratios and selection has been shown, e.g. for the evolution of viral infection (single versus coinfection) of host cells. Viruses from environments with higher virus–host ratios evolved more slowly than viruses from lower ratios (Turner, Cooper, and Lenski 1998). We did not investigate population dynamics of the host, virus, and virophage and test whether and how the observed evolution in the viruses affected population dynamics.

The observation that virus and virophage traits have evolved can have implications for the protective role of the virophage on the host population. This is because a decrease in virus exploitation by the virophage could reflect changes in the most common mode of virophage infection (reactivation versus coinfection). The virophage can integrate into the host genome upon infection and previous experiments have shown that ∼35 per cent of hosts carried integrated virophages 8 days post infection (Fischer and Hackl 2016). Since we found no cost to the host carrying integrated virophages, and thus no selection against integration (Supplementary Fig. S4), we expect that nearly all hosts in the virus–virophage treatment would have carried at least one copy of the integrated virophage halfway through the experiment. This change in the frequency of virophage-carrying hosts over time in the presence of the virus may shift the common mode of virophage infection toward reactivation, and thus a balanced virus and virophage production. With reactivation dominating, the virophages protection role for the host population is reduced. This proposed dynamic change in infection mode is related to horizontal or vertical transmission selection on bacteriophages and other parasites (Lipsitch, Siller, and Nowak 1996; Fellous and Salvaudon 2009; Shapiro, Williams, and Turner 2016) or mutualists (Roossinck 2011; Shapiro and Turner 2014), where the relative opportunity for horizontal versus vertical transmission has been shown to influence the evolution of symbiont effects on host fitness (e.g. (May and Anderson 1983; Ewald and Ewald 1987; Bull, Molineux, and Rice 1991; Bull 1994; Ebert 1994; Day 2001)). Generally, horizontal transmission from infected to uninfected cells favors increased

costs of infection for the host and more virulent phages or less beneficial mutualists, which leads to an increase in the rate of infection transfers. In our system, virophage transmission is only vertical (transmitted as provirophage by host replication) in the absence of giant virus infection, which is expected to be the major mode of transmission in wild host populations, as evidenced by the widespread occurrence of integrated virophages (Hackl et al. 2021). As the giant virus was present at the end of the experiment, we expect that virophage transmission was predominantly horizontal under the experimental conditions, which in contrast with phages, leaded to a decrease of virophage exploitation of the virus. It might be that the frequency of horizontal versus vertical transmission in this system oscillates over time due to the dual life cycle of the virophage.

Our experiments were started with non-clonal populations of giant viruses and virophages. The changes we observed could therefore be the result of selection on standing genetic variation and/or *de novo* mutations occurring in the chemostats. Similar to other experimental host–pathogen systems (Koskella and Brockhurst 2014; Frickel, Sieber, and Becks 2016; Shapiro, Williams, and Turner 2016), the present system had a high potential for coevolution of traits determining species interactions. In particular, the small genome size of the viruses compared to other microbes, the large population sizes (estimated harmonic mean population size from an independent chemostats experiment over 50 days for viruses $2*10^4$ DNA copies/ml and virophages $3*10^6$ DNA copies/ml; del Arco *unpublished data*), and the strong selection pressure (Nowak and May 2001) make *de novo* mutations likely to occur in this experiment.

The ddPCR protocol used to enumerate viruses and virophages in this study was designed to detect the strains used as free viruses, both for inoculation of the chemostats and for the assays. Therefore, there is a possibility that the number of viruses and virophages reported here may differ from the actual number, first, because viruses and virophages may have evolved to bind primers and/or probes less efficiently. We cannot exclude this possibility, although a single nucleotide substitution, the type of mutation we would expect in this experiment (Retel et al. 2022) should not reduce the efficiency of primer and probe binding. Secondly, *C. burkhardae* strain E4-10P carries several different endogenous virophages (EMALE) (Hackl et al. 2021), although there is currently little understanding of the conditions under which reactivation by CroV occurs (Berjón-Otero, Koslová, and Fischer 2019). Our ddPCR protocol does not allow us to determine whether other endogenous virophages have been reactivated. As we observed clear differences in host densities (Supplementary Fig. S1) when comparing host densities in the virus and virus–virophage treatments, we consider the possibility that other endogenous virophages influenced the evolutionary dynamics to be low. We can, however, not exclude it as characterizing the viral community at the end of the experiment is beyond the scope of this study.
Host-associated

microbes that mitigate the effects of pathogens on the host are common in nature and have been shown to shape evolutionary dynamics, reducing selection for resistance in the host (Oliver, Smith, and Russell 2014; Polin, Simon, and Outreman 2014; Ford et al. 2016). For example, the evolution of bacteriophages and satellite viruses showed that the coevolution of helper bacteriophages and satellite viruses reduces selection for bacterial resistance to the bacteriophage (Christie and Dokland 2012; Frígols et al. 2015; Krupovic, Kuhn, and Fischer 2016). Identifying and understanding the processes that allow interacting species with strong interdependence and mutual exploitation to persist is an ongoing challenge, and yet there are many such systems that persist over time. One evolutionary strategy that has been identified to facilitate persistence of such systems is reduced exploitation and reproduction. We showed in our experiments, that virus and virophage evolved reduced degrees of exploitation and replication, confirming the general theory on coexistence in tripartite systems and providing experimental evidence for processes that could facilitate persistence of protist–giant virus–virophage systems

Data availability

All data are uploaded to the KonData, the research data repository of the University of Konstanz (https://doi.org/10.48606/45).

Supplementary data

Supplementary data is available at *Virus Evolution Journal* online.

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References

Addy, H. S. et al. (2012) 'Loss of Virulence of the Phytopathogen Ralstonia Solanacearum through Infection by φRSM Filamentous Phages', *Phytopathology®*, 102: 469–77.

Berjón-Otero, M., Koslová, A., and Fischer, M. G. (2019) 'The Dual Lifestyle of Genome-integrating Virophages in Protists', *Annals of the New York Academy of Sciences*, 1447: 97–109.

Biggs, T. E. G., Huisman, J., and Brussaard, C. P. D. (2021) 'Viral Lysis Modifies Seasonal Phytoplankton Dynamics and Carbon Flow in the Southern Ocean', *The ISME Journal*, 15: 3615–22.

Bull, J. J. (1994) 'VIRULENCE', *Evolution; International Journal of Organic Evolution*, 48: 1423–37.

Bull, J. J., Molineux, I. J., and Rice, W. R. (1991) 'Selection of Benevolence in a Host-parasite System', *Evolution; International Journal of Organic Evolution*, 45: 875–82.

Christie, G. E., and Dokland, T. (2012) 'Pirates of the Caudovirales', *Virology, Special Issue: Viruses of Microbes*, 434: 210–21.

Day, T. (2001) 'Parasite Transmission Modes and the Evolution of virulence', *Evolution; International Journal of Organic Evolution*, 55: 2389–400.

del Arco, A., Fischer, M., and Becks, L. (2022) 'Simultaneous Giant Virus and Virophage Quantification Using Droplet Digital PCR', *Viruses*, 14: 1056.

del Arco, A., Woltermann, N., and Becks, L. (2020) Building up Chemostats for Experimental Eco-evolutionary Studies.

Duponchel, S., Fischer, M. G., and Condit, R. C. (2019) 'Viva Lavidaviruses! Five Features of Virophages that Parasitize Giant DNA Viruses', *PLOS Pathogens*, 15: e1007592.

Ebert, D. (1994) 'Virulence and Local Adaptation of a Horizontally Transmitted Parasite', *Science*, 265: 1084–6.

Ewald, P. W. (1987) 'Transmission Modes and Evolution of the Parasitism-Mutualism Continuuma', *Annals of the New York Academy of Sciences*, 503: 295–306.

Fellous, S., and Salvaudon, L. (2009) 'How Can Your Parasites Become Your Allies?', *Trends in Parasitology*, 25: 62–6.

Fenchel, T., and Patterson, D. J., (1988) '*Cafeteria Roenbergensis Nov. Gen., Nov. Sp., A Heterotrophic Microflagellate from Marine Plankton [WWW Document]*' <https://www.algaebase.org/search/genus/detail/?genus_id=43863> accessed 12 Feb 2021.

Fischer, M. G. et al. (2010) 'Giant Virus with a Remarkable Complement of Genes Infects Marine Zooplankton', *Proceedings of the National Academy of Sciences of the United States of America*, 107: 19508–13.

Fischer, M. G., and Hackl, T. (2016) 'Host Genome Integration and Giant Virus-induced Reactivation of the Virophage Mavirus', *Nature*, 540: 288–91.

Fischer, M. G., and Suttle, C. A. (2011) 'A Virophage at the Origin of Large DNA Transposons', *Science*, 332: 231–4.

Ford, S. A. et al. (2016) 'Microbe-mediated Host Defence Drives the Evolution of Reduced Pathogen Virulence', *Nature Communications*, 7: 13430.

Frickel, J., Sieber, M., and Becks, L. (2016) 'Eco-evolutionary Dynamics in a Coevolving Host–virus System', *Ecology Letters*, 19: 450–9.

Frickel, J., Theodosiou, L., and Becks, L. (2017) 'Rapid Evolution of Hosts Begets Species Diversity at the Cost of Intraspecific Diversity', *Proceedings of the National Academy of Sciences*, 114: 11193–8.

Frígols, B. et al. (2015) 'Virus Satellites Drive Viral Evolution and Ecology', *PLOS Genetics*, 11: e1005609.

Gaba, S., and Ebert, D. (2009) 'Time-shift Experiments as a Tool to Study Antagonistic Coevolution', *Trends in Ecology and Evolution*, 24: 226–32.

Gómez, P., and Buckling, A. (2011) 'Bacteria-Phage Antagonistic Coevolution in Soil', *Science*, 332: 106–9.

Gómez, P., Buckling, A., and Grover, J. (2013) 'Real-time Microbial Adaptive Diversification in Soil', *Ecology Letters*, 16: 650–5.

Guillard, R. R., and Ryther, J. H. (1962) 'Studies of Marine Planktonic Diatoms. I. Cyclotella Nana Hustedt, and Detonula Confervacea (Cleve) Gran', *Canadian Journal of Microbiology*, 8: 229–39.

Hackl, T. et al. (2021) 'Virophages and Retrotransposons Colonize the Genomes of a Heterotrophic Flagellate', *eLife*, 10: e72674.

Hampton, H. G., Watson, B. N. J., and Fineran, P. C. (2020) 'The Arms Race between Bacteria and Their Phage Foes', *Nature*, 577: 327–36.

Heilmann, S., Sneppen, K., and Krishna, S. (2010) 'Sustainability of Virulence in a Phage-Bacterial Ecosystem', *Journal of Virology*, 84: 3016–22.

Højsgaard, S. et al. (2020) Geepack: Generalized Estimating Equation Package.

Holt, R. D., and Hochberg, M. E. (1998) 'The Coexistence of Competing Parasites. Part II—Hyperparasitism and Food Chain Dynamics', *Journal of Theoretical Biology*, 193: 485–95.

Hothorn, T., Bretz, F., and Westfall, P. (2008) 'Simultaneous Inference in General Parametric Models', *Biometrical Journal*, 50: 346–63.

Huang, W. et al. (2017) 'Dynamical Trade-offs Arise from Antagonistic Coevolution and Decrease Intraspecific Diversity', *Nature Communications*, 8: 2059.

Koonin, E. V., and Krupovic, M. (2016) 'A Parasite's Parasite Saves Host's Neighbours', *Nature*, 540: 204–5.

Koskella, B., and Brockhurst, M. A. (2014) 'Bacteria-phage Coevolution as a Driver of Ecological and Evolutionary Processes in Microbial Communities', *FEMS Microbiology Reviews*, 38: 916–31.

Krupovic, M., Kuhn, J. H., and Fischer, M. G. (2016) 'A Classification System for Virophages and Satellite Viruses', *Archives of Virology*, 161: 233–47.

León, M., and Bastías, R. (2015) 'Virulence Reduction in Bacteriophage Resistant Bacteria', *Frontiers in Microbiology*, 6: 343.

Lipsitch, M., Siller, S., and Nowak, M. A. (1996) 'The Evolution of Virulence in Pathogens with Vertical and Horizontal Transmission', *Evolution*, 50: 1729–41.

Massana, R. *et al.* (2007) 'Crash of a Population of the Marine Heterotrophic Flagellate Cafeteria Roenbergensis by Viral Infection', *Environmental Microbiology*, 9: 2660–9.

May, R. M., and Anderson, R. M. (1983) 'Epidemiology and Genetics in the Coevolution of Parasites and Hosts', *Proceedings of the Royal Society of London. Series B*, 219: 281–313.

May, R. M., and Hassell, M. P. (1981) 'The Dynamics of Multiparasitoid-Host Interactions', *The American Naturalist*, 117: 234–61.

Morozov, A. Y., Robin, C., and Franc, A. (2007) 'A Simple Model for the Dynamics of A Host-parasite-hyperparasite Interaction', *Journal of Theoretical Biology*, 249: 246–53.

Nowak, M. A., and May, R. M. (2001) *Virus Dynamics: Mathematical Principles of Immunology and Virology*. Oxford University Press: Oxford, New York.

Oliver, K. M., Smith, A. H., and Russell, J. A. (2014) 'Defensive Symbiosis in the Real World – Advancing Ecological Studies of Heritable, Protective Bacteria in Aphids and Beyond', *Functional Ecology*, 28: 341–55.

Paez-Espino, D. et al. (2019) 'Diversity, Evolution, and Classification of Virophages Uncovered through Global Metagenomics', *Microbiome*, 7: 1–4.

Parratt, S. R., and Laine, A.-L. (2016) 'The Role of Hyperparasitism in Microbial Pathogen Ecology and Evolution', *The ISME Journal*, 10: 1815–22.

Piel, D. et al. (2022) 'Phage–host Coevolution in Natural Populations', *Nature Microbiology*, 7: 1075–86.

Pinheiro, J. et al. (2017) 'Nlme: Linear and Nonlinear Mixed Effects Models', R Package Version 3.1-131. 69, 18637.

Polin, S., Simon, J.-C., and Outreman, Y. (2014) 'An Ecological Cost Associated with Protective Symbionts of Aphids', *Ecology and Evolution*, 4: 826–30.

R Core Team, V. 4. 1. 1. 2020. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Retel, C. et al. (2022) 'Strong Selection and High Mutation Supply Characterize Experimental Chlorovirus Evolution', *Virus Evolution*, 8: veac003.

Roossinck, M. J. (2011) 'The Good Viruses: Viral Mutualistic Symbioses', *Nature Reviews, Microbiology*, 9: 99–108.

Rosenzweig, M. L., and MacArthur, R. H. (1963) 'Graphical Representation and Stability Conditions of Predator-Prey Interactions', *The American Naturalist*, 97: 209–23.

Roux, S. et al. (2023) 'Updated Virophage Taxonomy and Distinction from Polinton-like Viruses', *Biomolecules*, 13: 204.

RStudio Team, V. 2020. RStudio: Integrated Development for R. Boston, MA: RStudio, PBC. http://www.rstudio.com/.

Santander, J., and Robeson, J. (2007) 'Phage-resistance of Salmonella Enterica Serovar Enteritidis and Pathogenesis in Caenorhabditis Elegans is Mediated by the Lipopolysaccharide', *Electronic Journal of Biotechnology*, 10.

Scheuerl, T. et al. (2020) 'Bacterial Adaptation is Constrained in Complex Communities', *Nature Communications*, 11: 754.

Schoenle, A. (2020) 'Global Comparison of Bicosoecid Cafeteria-like Flagellates from the Deep Ocean and Surface Waters, with

Reorganization of the Family Cafeteriaceae', *European Journal of Protistology*, 73: 125665.

Shapiro, J. W., and Turner, P. E. (2014) 'The Impact of Transmission Mode on the Evolution of Benefits Provided by Microbial Symbionts', *Ecology and Evolution*, 4: 3350–61.

Shapiro, J. W., Williams, E. S. C. P., and Turner, P. E. (2016) 'Evolution of Parasitism and Mutualism between Filamentous Phage M13 and Escherichia Coli', *PeerJ*, 4: e2060.

Stahl, E. A. et al. (1999) 'Dynamics of Disease Resistance Polymorphism at the Rpm1 Locus of Arabidopsis', *Nature*, 400: 667–71.

Turner, P. E., Cooper, V. S., and Lenski, R. E. (1998) 'Tradeoff between Horizontal and Vertical Modes of Transmission in Bacterial Plasmids', *Evolution*, 52: 315–29.

Wodarz, D. (2013) 'Evolutionary Dynamics of Giant Viruses and Their Virophages', *Ecology and Evolution*, 3: 2103–15.

Xiao, C. et al. (2017) 'Cryo-EM Reconstruction of the Cafeteria Roenbergensis Virus Capsid Suggests Novel Assembly Pathway for Giant Viruses', *Scientific Reports*, 7: 5484.

Yau, S. et al. (2011) 'Virophage Control of Antarctic Algal Host-virus Dynamics', *Proceedings of the National Academy of Sciences of the United States of America*, 108: 6163–8.

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