FOCUSED TOPIC

Multiyear Time-Shift Study of Bacteria and Phage Dynamics in the Phyllosphere*

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ABSTRACT: Coevolution shapes diversity within and among populations but is difficult to study directly. Time-shift experiments, where individuals from one point in time are experimentally challenged against individuals from past, contemporary, and/or future time points, are a powerful tool to measure coevolution. This approach has proven useful both in directly measuring coevolutionary change and in distinguishing among coevolutionary models. However, these data are only as informative as the time window over which they were collected, and inference from shorter coevolutionary windows might conflict with those from longer time periods. Previous time-shift experiments from natural microbial communities of horse chestnut tree leaves uncovered an apparent asymmetry, whereby bacterial hosts were more resistant to bacteriophages from all earlier points in the growing season, while phages were most infective to hosts from only the recent past. Here, we extend the time window over which these infectivity and resistance ranges are observed across years and confirm that the previously observed asymmetry holds over longer timescales. These data suggest that existing coevolutionary theory should be revised to include the possibility of differing models for hosts and their parasites and examined for how such asymmetries might reshape the predicted outcomes of coevolution.

Keywords: time shift, bacteria, bacteriophage, coevolution, horse chestnut tree.

Introduction

The evolution of life is shaped by selection from the abiotic and biotic environments in which populations co-occur. Understanding how species interactions shape evolutionary and coevolutionary trajectories is critical in predicting community diversity, stability, and function. For bacteria, a key biotic factor shaping evolution and diversity is selection by bacteriophage viruses (or phages; Scanlan 2017; Breitbart et al. 2018; Morella et al. 2018). For lytic phages that infect and kill their host cells, there is expected to be strong selection for bacterial resistance (Koskella and Brockhurst 2014). Our understanding of the myriad ways that resistance against phage infection and/or phage replication within cells can occur continues to expand (Azam and Tanji 2019; Mutalik et al. 2020), and regardless of mechanism there is strong evidence from both experimental (e.g., Lenski and Levin 1985; Buckling and Rainey 2002; Broniewski et al. 2020) and natural (e.g., Held and Whitaker 2009; Koskella 2013; Seed et al. 2014) systems that resistance evolves readily under phage-mediated selection, including as a result of phage therapy (Oechslin 2018). The diversity of resistance mechanisms that exists (recently reviewed in Hampton et al. 2020) and the speed at which resistance evolves and spreads in nature emphasize that phage-mediated selection is an important force in shaping bacterial populations and communities. However, insight into the dynamics of these systems requires cross-scale analyses, including over longer timescales that are typically not possible in the laboratory and prove challenging in natural systems.

Theory predicts that the impact of species interactions on diversity (both over space and time) will depend on the coevolutionary dynamics underlying the interactions (recently reviewed in Hall et al. 2020). Broadly, coevolution can be usefully studied by looking at the level of specificity for the interaction (generalists vs. specialists; e.g., Flores

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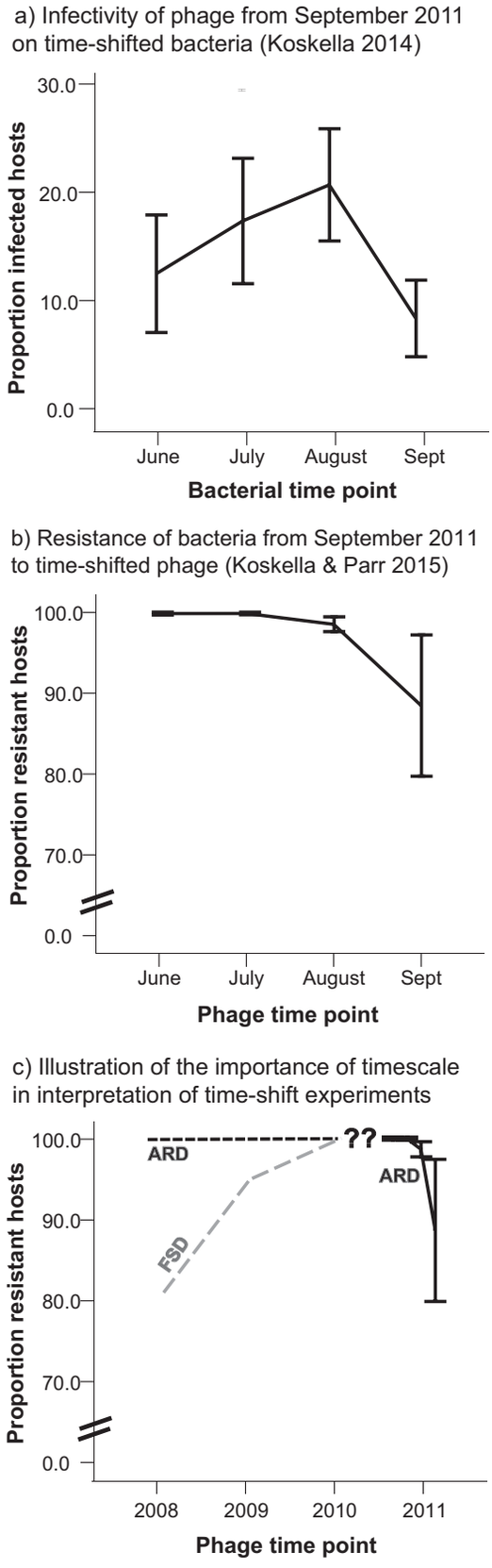
et al. 2011), the genomic underpinnings and impacts of the interaction (e.g., Papkou et al. 2019), the speed at which coevolution occurs (e.g., Brockhurst et al. 2003), and/or the phenotypic adaptations resulting from selection in each population (Lopez Pascua et al. 2014). The spatial and temporal patterns that emerge when studying coevolution are often dichotomized as either directional or fluctuating selection (e.g., Gómez and Buckling 2011), and two models are typically considered to describe these modes of coevolution (although note that others exist; e.g., Fenton et al. 2009). The arms race dynamics (ARD) model suggests that resistance and counterresistance adaptations tend to reach fixation in the population before the next set of adaptations evolve (Gandon 2002; Woolhouse et al. 2002). This leads to the simplified prediction that resistance evolved at one time point will be effective against all past parasite types, and any counteradaptation in the parasite population will allow parasites to infect all previously resistant hosts. In contrast, fluctuating selection dynamics (FSD) are generally assumed when the resistance of a host depends on the relative prevalence of parasite genotypes in the local environment, and resistance at one time point can be ineffective against both parasites from the past and those from the future if a different parasite genotype was or becomes dominant (Barrett 1988; Lively and Apanius 1995; Ebert and Hamilton 1996). While the ARD model is generally considered to be driven by generalist phenotypes, with expanding parasite host range as new counteradaptations arise that are effective against a wider and wider range of previously evolved resistances and expanding host resistance ranges against previous parasite types, the FSD model tends to assume tight genotype-by-genotype specificity, where host genotypes are not inherently different in their level of resistance but rather have different resistance profiles (although see Best et al. 2017). As such, these models can lead to different predictions regarding how coevolution shapes diversity (Hall et al. 2020) and local adaptation (Lively 1999; Gandon 2002), and they are typically assumed to reflect different infection genetics of the system (reviewed in Brockhurst and Koskella 2013).

Time-shift experiments are a powerful tool for measuring coevolutionary change and differentiating among these dynamics (Gaba and Ebert 2009; Blanquart and Gandon 2013). For any organism that can be resurrected from the past (e.g., by surviving in frozen stocks [Buckling and Rainey 2002] or having resting stages trapped in sediment [Decaestecker et al. 2007]), it is possible to test interactions with antagonists across time, where populations from the past, present, and future are challenged against one another and the outcome of the interactions are compared. Predictions of coevolutionary change can in this way be tested directly, for example, by asking whether hosts are more resistant to parasites from the past and less resistant to those from the future. Importantly, if these time shifts are performed across multiple past populations and multiple future populations, the contrasting predictions of ARD and FSD in durability of infectivity and resistance through time can be tested (Gaba and Ebert 2009). Here, we would predict that FSD results in hosts that are more resistant to parasites from the recent past but susceptible to parasites from further back in time. In contrast, ARD would result in hosts that are generally resistant to all past parasites and generally sensitive to all future parasite populations tested. A critical drawback of interpreting data from time-shift experiments is the difficulty in ruling out FSD when ARD-like patterns are observed because of the possibility that too short of a window of time was observed (Gaba and Ebert 2009).

Recent work from bacteria-phage systems in vitro has highlighted the fluidity among these models of coevolution. There is now evidence that coevolutionary dynamics can be shaped not only by the bacterial and phage strains being tested (a genotype × genotype interaction, as observed by Betts et al. 2014) but also by the environmental context. For example, experimentally (co)evolving populations with higher nutrient availability were found to be more in line with ARD than those from lower-nutrient environments, likely due to the reduced costs of resistance under higher nutrients (Lopez Pascua et al. 2014). Similarly, experimental microcosms that were mixed via shaking were found to undergo ARD, unlike those left static, here suggesting that increased selection due to higher phage contact rates can shift dynamics from FSD toward ARD (Gómez et al. 2015). In addition to these dynamics being shaped by the environmental context, as predicted by theory (e.g., Lopez Pascua et al. 2014; Gómez et al. 2015), they may also themselves change over the course of a coevolutionary interaction. One experimental coevolution study that was conducted over slightly longer timescales in the lab than is typical revealed that while coevolution initially resembled ARD, it began to resemble FSD over time, presumably after the mutations of major effect had been selected on and costly mutations were lost (Hall et al. 2011). In these cases, however, the shift from one dynamic to another across treatments or time was observed to be symmetrical (i.e., observed across both the bacterial time shift and the phage time shift).

In contrast to results from the laboratory, data from environmental bacteria and phage interactions are less likely to readily conform to simplified models. This is unsurprising given the complexities of the environments being sampled and, often, the inability to track coevolutionary change within lineages. Work from the phyllosphere (leaves) of the perennial horse chestnut tree (*Aesculus hippocastanum*), however, has demonstrated that despite the likely many other selective pressures exerted on bacterial



communities, lytic phages are well adapted to their local host populations/communities (Koskella et al. 2011) and can select for bacterial resistance to phages over time (Koskella 2013). Using a series of time-shift experiments within a single growing season, this system was used to measure phage infectivity and bacterial resistance when each was challenged against antagonists from either the same month or earlier months in the season. In the first set of crosses, where phage infectivity was measured on time-shifted bacterial communities, phage infection patterns were found to be most similar to FSD, where phages were most infective to bacteria from the recent past (a month earlier) but were less infective to hosts from earlier in the season (Koskella 2014). Surprisingly, when the reciprocal time shift was performed across the same communities but focusing on bacterial resistance against time-shifted phages from earlier in the season, the dynamics were observed to be more in line with ARD, where bacteria were resistant to all phages from earlier in the season (Koskella and Parr 2015). One potential explanation for this observed asymmetry in coevolutionary dynamics is that the time window (one season) captured loss of phage infectivity but not loss of bacterial resistance, for example, because of longer relative generation times of bacteria or different strengths or efficacy of selection acting on bacterial populations relative to phages (fig. 1; Gaba and Ebert 2009).

To determine whether this previously observed asymmetry could be explained by the single-season time window, we performed time-shift experiments to compare phage infectivity and bacterial resistance against antagonists that were collected in the same nominal month but from across 4 years. Experimental crosses were done only within the same (sympatric) tree and from the same eight trees used previously. We predicted that if the observed asymmetry was a consequence of too short of a time window,

Figure 1: Importance of scale in time-shift experiments. Previous results from time-shift experiments performed over a single growing season and how these might change over longer time scales. *a*, Results from Koskella (2014), in which phages from September (across the same set of eight trees used in the current study) were time shifted against bacteria from earlier in the season. Results suggest that phage infectivity is short-lived and decreases against bacteria from further in the past as predicted under the fluctuating selection dynamics (FSD) model. *b*, Results from Koskella and Parr (2015), in which bacteria from September (across the same set of eight trees used in the current study) were time shifted against phages from earlier in the season. In this case, bacteria were found to be consistently more resistant to phages from the past, even those from the start of the season, in line with predictions from the arms race dynamics (ARD) model. *c*, Illustration of how the conclusions reached from time-shift data are limited by the window of time in which the shift was performed. The dashed lines illustrate how a pattern considered to reflect ARD in one window (i.e., within a season) could either remain consistent with this model or become more in line with FSD by extending the time window (in this case across multiple years).

then bacterial resistance should decrease against phages from earlier years relative to phages from either the contemporary time point or the more recent past. Instead, we again observed surprising asymmetry, whereby bacterial hosts tended to be more resistant to phages from the past and less resistant to phages from the future, while phages were not consistently more infective to bacteria from the past. This suggests that bacteria-phage dynamics can be detected even after the leaves they inhabit have dropped and new leaves have emerged. The results emphasize that existing theory for coevolutionary dynamics likely needs to be reexamined to incorporate the possibility that asymmetries in selection and/or genetic underpinnings can lead to disparate patterns between host and parasite populations.

Methods

Sample Collection

Bacteria and phages were isolated from whole leaves as previously described in Koskella (2014) and Koskella and Parr (2015). Briefly, two leaves from the same branch were collected over 4 years from the same set of eight individual horse chestnut trees (*Aesculus hippocastanum*; $N = 64$ leaves) located in an urban park in Oxfordshire, United Kingdom. Sampling occurred once at the end of the growing season on the following dates: September 15, 2011; August 19, 2012; September 16, 2013; and September 3, 2014. Leaves were immediately processed in the lab for long-term freezer storage to preserve the leaf and its microbes. Individual leaves were surface sterilized and then stored in buffer at −20°C.

After all leaf sampling was complete, randomly selected leaves were rapidly thawed and then homogenized with sterile ceramic beads using a FastPrep 24 system (MP Biomedicals). The homogenate was diluted and plated onto King's broth (KB) 1.2% agar plates using sterile glass beads, then incubated for 48 h at 28°C. Forty-eight colonies were picked per plate, incubated in KB overnight at 28°C, then stored with glycerol in buffer at −80°C. This was repeated until all of the leaves were processed, resulting in 96 bacterial isolates per tree in a given year. To extract phage, the remaining leaf homogenate was centrifuged and passed through a 0.45-μm filter to remove any bacteria, then stored at 4°C in the dark. Leaf homogenate from the two leaves was bulked together to give one phage inoculum per tree per year, as phage infectivity is similar across leaves from the same tree in this system (Koskella et al. 2011).

Time-Shift Experiments

Each of the 96 bacterial isolates per tree/year was crossed with phage filtrate from each of the 4 years from the same (sympatric) tree. All crosses used soft agar overlays, as described previously in Koskella et al. (2011), but were modified for a 24-well plate. Briefly, each bacterial isolate was grown from freezer stocks overnight at 28°C, then mixed into warm soft agar and pipetted on top of a hard agar base in each well of a 24-well plate. Once cooled, 10 μL of phage filtrate was spotted into each well, and the plates were incubated overnight at 28°C. Two control wells were included for each bacterial isolate by spotting sterile water rather than phage filtrate. A bacterial host was considered susceptible if clearances (plaques) in the lawn were visible and the absence of bacterial growth overlapped with where the phage inoculum had been spotted.

Phage Clone Isolations

Individual phages were isolated by combining susceptible host cells with an agar plug from the observed plaque, then coculturing overnight at 28°C. We then added 100 μL of chloroform to the coculture to kill bacteria, and tubes were centrifuged at 13,000 rpm for 3 min to pellet cell debris. The phage supernatant was removed and stored at 4°C in the dark. Successfully isolated phages were then single-plaque purified again on the originally sensitive host, cocultured for 24 h, and then filter purified to generate a high titer inoculum of each single phage. For the 20 phages that were successfully isolated and amplified, we then tested their infectivity against all sympatric hosts from past, contemporary, or future time points using the same soft agar overlay approach.

Sequencing

After all crosses were completed, each of the 3,072 bacterial isolates were identified using Sanger sequencing of the 16S rRNA gene, using primers 63f (5′-CAGGCCTAACACATGCAAGTC-3′; Marchesi et al. 1998) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′; Lane 1991). Sequences were processed using Geneious 6.4 and blasted using the National Center for Biotechnology Information (NCBI) nucleotide database. Species-level identification was made using the highest Geneious grade, or when the grade tied or further classification was not possible, the isolate was identified to the genus level.

Statistical Analyses

All statistical tests were conducted in R (ver. 4.0.0; R Development Core Team 2020). To examine the relative abundances of dominant bacterial genera (as many samples were identified only to the genus level), we separately modeled the relative abundance of *Pseudomonas*, *Erwinia*, and *Pantoea*

as a function of tree, year, and their interaction with a generalized linear model with a binomial response and logit link using the glm function from the stats package in R (ver. 4.0.0; Bates et al. 2015). To examine the coevolutionary dynamics, we ran a series of generalized linear mixed effects models with a binomial response and logit link on infection outcomes from the time-shift experiments using the glmer function from the lme4 package in R (ver. 4.0.0; Bates et al. 2015). Goodness of fit for logistic models was determined with the Hosmer-Lemeshow test using the hoslem.test function from the ResourceSelection package in R (ver. 4.0.0; Lele et al. 2019). We ran analyses on the outcomes of sympatric time-shift assays looking at (1) all bacterial hosts and phages (the metacommunity), (2) only the dominant bacterial genera and their phages, and (3) only the individually isolated phage clones on their major host genus or genera. Data on either bacterial resistance against time-shifted phages (analyses 1 and 2) or phage infectivity against time-shifted bacteria (analyses 1 and 3) were used to distinguish between models of coevolutionary dynamics. The first analysis was run across all 4 years; the second analysis was run within individual years. Last, we ran Tukey's post hoc tests using the glht function from the multcomp package in R (ver. 4.0.0; Hothorn et al. 2008) to determine whether infectivity or resistance significantly changed between host/parasite time points and in what direction. In the third analysis, focusing on individual phage clones, we accounted for the possibility that the same phages were repeatedly sampled by grouping the phages by tree and year and treating individual isolates as technical replicates. This resulted in four phage groups: tree 1 in 2013 ($N = 11$), tree 1 in 2014 ($N = 1$), tree 2 in 2014 ($N = 5$), and tree 6 in 2014 ($N = 3$). Tree 1 in 2014 had only one replicate and was discarded from our analyses. To control for changes in community structure over time, we ran our model series only on the host genera that made up the greatest proportion of infections per phage group: *Pseudomonas* (50%–78%) for tree 1 in 2013, *Pantoea* (61%–81%) for tree 2 in 2014, and both *Erwinia* (32%–54%) and *Pseudomonas* (38%) for tree 6 in 2014. For additional experimental and statistical methods descriptions, see the supplemental PDF, available online.

Results

In the first time-shift assay, we examined 12,288 pairwise sympatric host-phage interactions. Each of our 3,072 isolated hosts were tested against their four sympatric phage filtrates, with 163 hosts (5.3%) being susceptible to at least one phage sample. There were 2,881 hosts that were successfully sequenced and identified to at least the genus level (with 155 not successfully sequenced and 36 with no significant matches in NCBI). We observed a high level of variation in susceptibility of the hosts that were sequenced, with four identified genera—*Erwinia*, *Pantoea*, *Pseudomonas*, and *Stenotrophomonas*—being susceptible to at least one phage sample and with phage samples infecting an average of 1.71 (0.66 SD) host genera. Of the sequenced bacterial isolates, 687 (22.36%) were *Erwinia*, with 21 (3.06%) being susceptible to phage; 694 (22.59%) were *Pantoea*, with 6 (0.86%) being susceptible to phage; and 1,278 (41.60%) were *Pseudomonas*, with 96 (7.51%) being susceptible to phage. Neither the tree host nor year of collection (or their interaction) explained significant variation in the relative abundance of these dominant genera within their communities ($P > .05$; fig. 2).

The average host range for the infective phage samples was 8.76 (10.13 SD) host isolates, with some phage samples (from tree 1 in 2013 and 2014) infecting as many as 45 hosts and three samples (from tree 4 in 2011 and 2013 and tree 8 in 2011) never infecting any hosts. There was considerable variation in host susceptibility and phage infectivity across the four sampled years, with a low of 10 (1.3%) susceptible individual hosts from 2011 and a high of 55 (7.2%) susceptible individual hosts from 2013. Phage samples had average host ranges from a low of 5.3 (4.37 SD) to a high of 12.0 (11.94 SD) individual hosts. For additional information on general bacterial resistance and phage infectivity, see the supplemental PDF.

In a separate time-shift assay, we tested 1,152 bacterial hosts against 20 phage isolates (384 sympatric hosts per phage), which were later bulked into three phage groups for analysis as described above. Bacteria belonging to five genera (*Erwinia*, *Panteoa*, *Pseudomonas*, *Rahnella*, and *Staphylococcus*) and one class (Gammaproteobacteria, which includes all of the previously mentioned genera except *Staphylococcus*) were susceptible to at least one phage isolate. Of the 1,152 tested sympatric hosts, 371 (32.20%) were susceptible to an average of 0.65 (1.44 SD) individual phage isolates, with all phage isolates infective to at least one host. On average, the individual phage isolates infected 37.40 (15.06 SD) individual hosts and 4.65 (0.67 SD) identified host genera, and the phage groups infected 187.0 (145.75 SD) individual hosts and 5.25 (0.50 SD) identified host genera.

Coevolutionary Dynamics in the Metacommunity

We used a series of generalized linear mixed effects models to investigate the coevolutionary dynamics for bacterial hosts and phages. For host resistance (fig. 3*a*), we found a significant effect of host year (host year: estimate $= -0.61$, SE $= 0.12, z = -5.11, P \ll .0001$) and an interaction between year and phage time shift (host year × phage time shift: estimate $= -0.12$, SE $= 0.055$, $z = -2.26$, $P = .024$) but no main effect of phage time shift ($P = .98$). To account

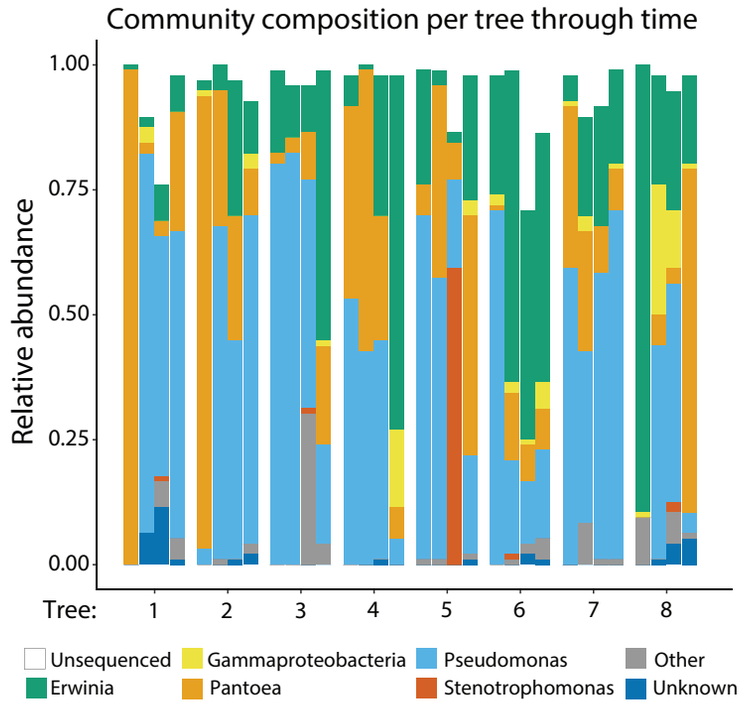


Figure 2: Relative abundances of dominant bacterial genera are not dependent on either tree or year. Taxa that comprise >2% of all bacterial samples, as well as bacteria that were not conclusively identified by sequencing ("unknown," 1.2% of all samples), are represented individually by colored bars; taxa that comprise <2% of all bacterial samples (13 in total) are represented in the "other" category by gray bars. Bacterial samples that were not sequenced successfully ("unsequenced") are represented by white bars, such that each column ranges the full 0%–100% relative abundance with the white space representing the members of that community that could not be sequenced. Columns are grouped by tree and ordered within tree by year going from left (2011) to right (2014). The relative abundances of the three dominant genera—*Erwinia*, *Pantoea*, and *Pseudomonas*—are not explained by tree, year, or their interaction ($P > .05$).

for the potential of phage decay in storage that would result in reduced infectivity of earlier phage inocula, we reran the model excluding data from 2011 phage samples and obtained similar results. After excluding all data from 2011 hosts, given the overwhelming resistance observed, we found a significant effect of host year (host year: estimate $= -0.38$, SE $= 0.138$, $z = -2.75$, $P < .01$), suggesting that hosts are generally becoming somewhat less resistant over time, and no main effect of phage time shift ($P = .18$) or interaction between the two ($P = .91$). Within separate host years, phage time shift had a significant effect on resistance of hosts from 2012 (phage time shift: estimate $= -0.46$, SE $= 0.208$, $z = -2.22$, $P = .027$), 2013 (phage time shift: estimate $= -0.33$, SE $= 0.162$, $z = -2.03$, $P = .043$), and 2014 (phage time shift: estimate $= -0.41$, SE $= 0.123$, $z = -3.33$, $P < .001$) but had no effect on hosts from 2011 ($P = .33$). Using Tukey's post hoc tests, we found that hosts from 2013 and 2014 tended to be more resistant to phages from past time points and less resistant to phages from contemporary and/or future time points (table S1; tables S1–S3 are available online), consistent with ARD. Hosts from 2012 were marginally more resistant to past phages than to future phages ($P = .097$; table S1).

For phage infectivity across all years (fig. 3*b*), we found an effect of phage sample year (phage year: estimate $= 0.66$, SE $= 0.098$, $z = 6.79$, $P \ll .0001$), suggesting that phages are generally increasing in infectivity over time, and a marginal effect of host time shift (host time shift: estimate $= 0.26$, SE $= 0.152$, $z = 1.73$, $P = .084$) but no interaction ($P = .78$). After excluding all data from 2011 phages, we found similar results but found no effect of host time shift ($P = .54$). Within years, host time shift had a significant effect on infectivity for phages from 2011 (host time shift: estimate $= 0.47$, SE $= 0.190$, $z = 2.50$, $P = .012$), 2013 (host time shift: estimate $= 0.34$, SE $= 0.138$, $z = 2.46$, $P = .014$), and 2014 (host time shift: estimate $= 0.27$, SE $= 0.114$, $z = 2.39$, $P = .017$) but had no effect for phages from 2012 ($P = .41$). Using Tukey's post hoc tests, we found that phages from 2013 and 2014 tended to be less infective on far-past hosts than on contemporary hosts (table S1), consistent with FSD. Phages from 2011 were more infective on future hosts

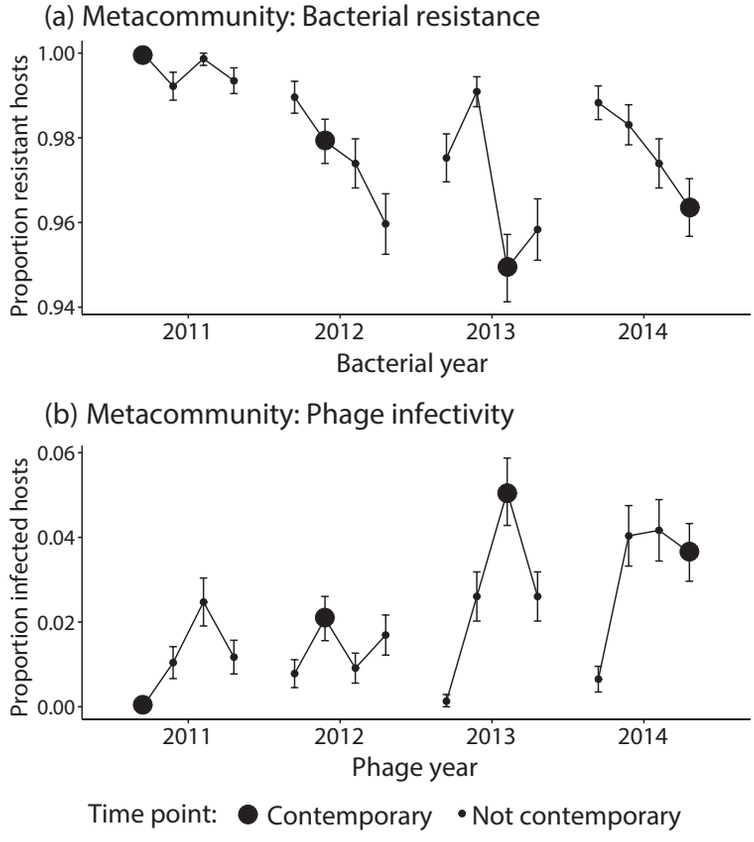


Figure 3: Time shifts reveal that host resistance follows arms race dynamics (*a*), while phage infectivity follows fluctuating selection dynamics (*b*) across years. Individual bacterial hosts ($N = 3{,}072$) were challenged in time-shift assays with sympatric phage communities ($N = 32$) across four annual time points. The *x*-axes denote the contemporary year of the host (*a*) or phage (*b*). The *y*-axes denote the proportion of resistant hosts (*a*) and infected hosts (*b*). For readability, *y*-axes do not show the full range of 0–1.0; also note that *a* shows the upper range of 0.94–1.0, while *b* shows the lower range of 0.0–0.06. Average outcomes for pairwise interactions are represented by data points, grouped by contemporary year, and are ordered by the antagonist's time point from left (2011) to right (2014), with the contemporary demarcated by the largest data point. Error bars indicate the standard error calculated with the Jeffreys interval for extreme probabilities ($\mathrm{SE} = (\widehat{p}[1-\widehat{p}]/[n+1])^{1/2}$, where $\widehat{p} = (x + 1/2)/(n + 1)$, x is the number of successes, and n is the number of trials). *a*, Hosts from 2013 and 2014 are more resistant to past phages, consistent with arms race dynamics, while hosts from 2012 are marginally more resistant (Tukey's post hoc test; table S1). *b*, Consistent with fluctuating selection dynamics, phages from 2013 and 2014 are less infective on far-past hosts than on contemporary hosts, while phages from 2011 are more infective on future hosts (Tukey's post hoc test; table S1).

than on contemporary hosts, which, while not frequently observed in studies, is also indicative of FSD (table S1).

Host Resistance Dynamics across Dominant Genera

We used a similar series of generalized linear mixed effects models to investigate host resistance in the dominant genera *Erwinia*, *Pantoea*, and *Pseudomonas* (fig. 4). For host resistance across all three genera, we found a main effect of host collection year (host year: estimate $= -0.69$, SE $=$ 0.120, $z = -5.73$, $P \ll .0001$) and its interaction with phage time shift (phage time shift × host year: estimate $=$ -0.15, SE $= 0.057$, $z = -2.64$, $P < .01$). We also found a significant effect of genus (genus: estimate $= -0.11$, SE $= 0.030$, $z = -3.81$, $P < .001$) and a marginal effect of phage time shift (phage time shift: estimate $= -0.50$, SE $= 0.281$, $z = -1.79$, $P = .074$) but no interaction ($P = .49$). For *Erwinia* (fig. 4*a*), resistance marginally depended on year (host year: estimate $= 0.48$, SE $= 0.278$, $z = 1.71$, $P = .087$), but we found no effect of phage time shift ($P = .14$) or an interaction ($P = .12$). Within each year, we found no effect of phage time shift on *Erwinia* resistance ($P > .05$). For *Pantoea* (fig. 4*b*), we found a significant effect of year on resistance (host year: estimate $=$ -2.22, SE $= 0.827$, $z = -2.69$, $P < .01$) but no effect of phage time shift ($P = .30$) or interaction ($P = .44$). Within each year, we found no effect of phage time shift on *Pantoea* resistance ($P > .05$). For *Pseudomonas* (fig. 4*c*), we found a significant effect of host year on resistance (host year: estimate $= -0.84$, SE $= 0.133$, $z = -6.29$,

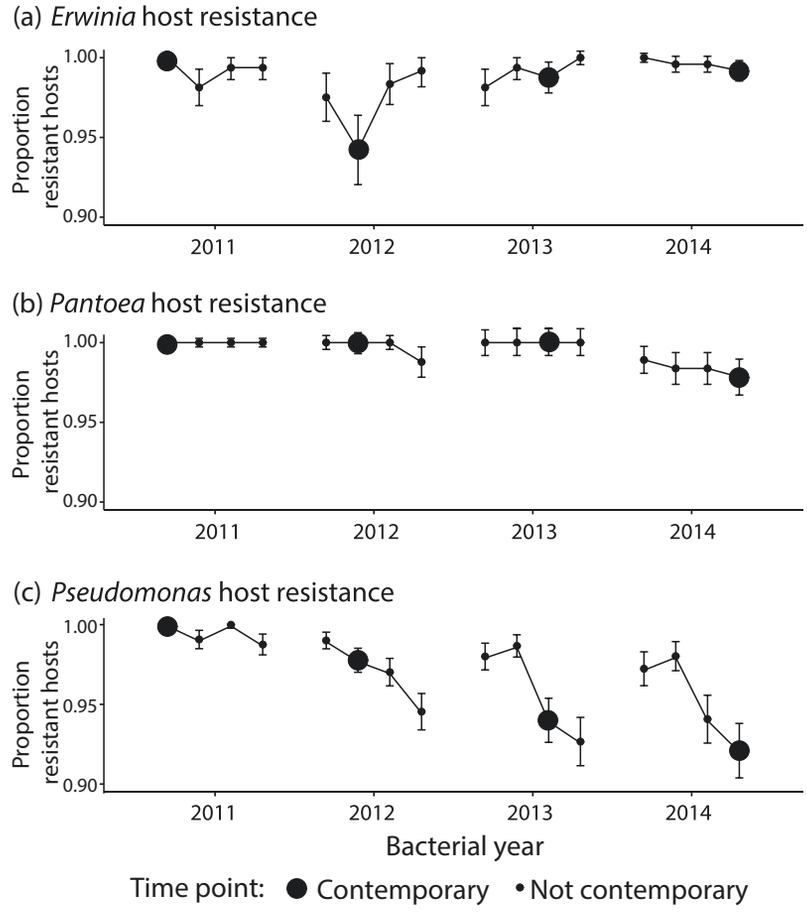


Figure 4: Host resistance follows arms race dynamics in *Pseudomonas* (*c*) but not in *Erwinia* (*a*) or *Pantoea* (*b*). Bacterial isolates identified as *Erwinia* ($N = 687$), *Pantoea* ($N = 694$), and *Pseudomonas* ($N = 1{,}278$) were separately examined in terms of resistance against sympatric phage communities ($N = 32$) across the four annual time points. The *x*-axes denote the contemporary host year; the *y*-axes denote the proportion of resistant hosts. For readability, *y*-axes show only the range of 0.90–1.0. Average outcomes for pairwise interactions are represented by data points, grouped by contemporary year, and are ordered by the phage time point from left (2011) to right (2014), with the contemporary demarcated by the largest data point. The error bars represent the standard error calculated with the Jeffreys interval for extreme probabilities ($\mathrm{SE} = (\widehat{p}[1-\widehat{p}]/[n+1])^{1/2}$, where $\widehat{p} = (x + 1/2)/(n + 1)$, x is the number of successes, and n is the number of trials). Host resistance in *Erwinia* (*a*) and *Pantoea* (*b*) was not dependent on phage time shift within individual years ($P > .05$). *Pseudomonas* (*c*) collected in 2013 and 2014 were more resistant to past phages, consistent with arms race dynamics.

$P \ll .0001$), again suggesting that hosts are becoming generally less resistant over time, and we found no effect of phage time shift and a marginal interaction (phage time shift × host year: estimate $= -0.13$, SE $= 0.068$, $z = -1.84$, $P = .066$). Within each year, we found a significant effect of phage time shift for *Pseudomonas* from 2012 (phage time shift: estimate $= -0.56$, SE $= 0.160$, $z = -3.49$, $P < .001$), 2013 (phage time shift: estimate $= -0.64$, SE $= 0.179$, $z = -3.61$, $P < .001$), and 2014 (phage time shift: estimate $= -0.45$, SE $= 0.165$, $z = -2.74$, $P < .01$) but not from 2011 ($P = .30$). Using a Tukey's post hoc test, we found that *Pseudomonas* hosts from 2013 and 2014 tend to be more resistant to phages from the past than to phages from the future (table S2), consistent with ARD and our metacommunity-wide results, but we found no significant differences between phage time points for *Pseudomonas* from 2012 ($P > .05$; table S2).

Infectivity Dynamics for Individually Isolated Phages

We used generalized linear mixed effects models to identify patterns in infectivity of the phage isolate groups (tree 1 in 2013, tree 2 in 2014, and tree 6 in 2014) on only their major host genus or genera (fig. 5). Across all phage groups, the effect of host time shift on infectivity depended on sampling year (host time shift × phage year: estimate $= 1.49$, SE $= 0.184$, $z = 8.08$, $P \ll .0001$) and on the phage group (host time shift × phage group: estimate $= 0.58$, SE $= 0.081$, $z = 7.20$, $P \ll .0001$). For the phages from tree 1 in 2013 on *Pseudomonas* hosts

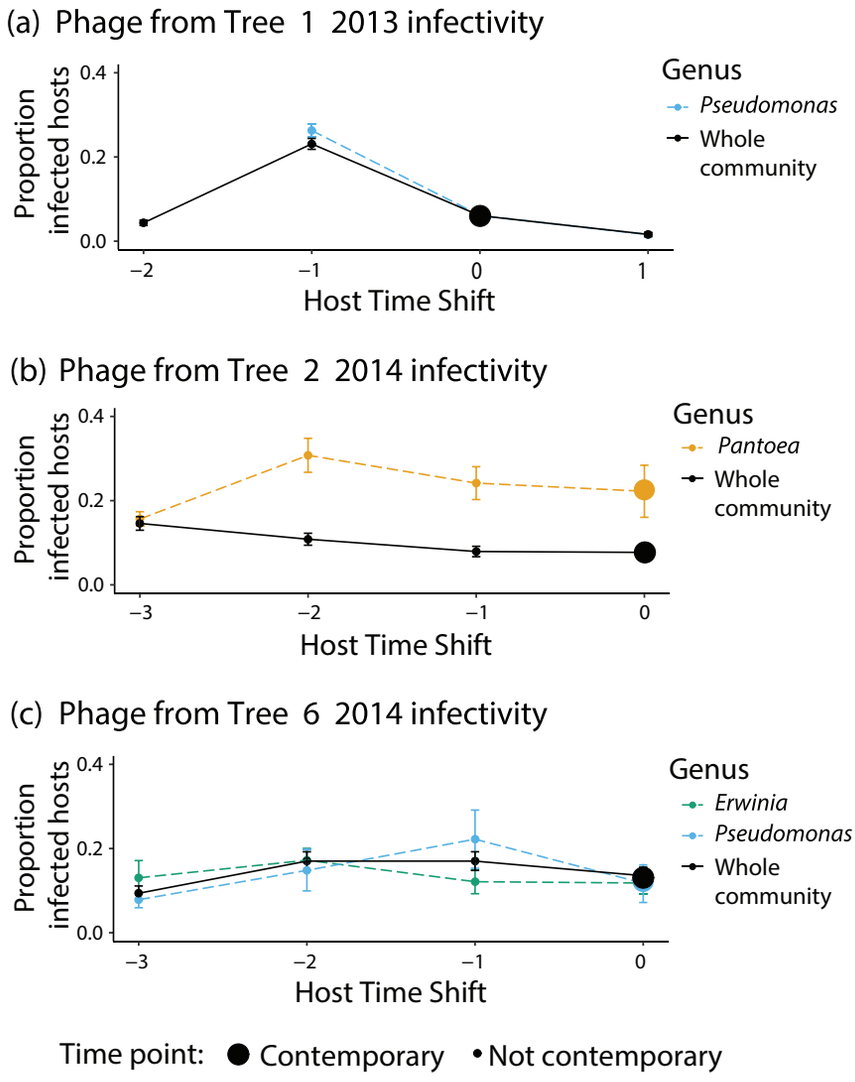


Figure 5: Grouped isolated phages demonstrate apparent fluctuating selection dynamics and arms race dynamics on major host genera. Grouped isolated phages from tree 1 in 2013 ($N = 11$; *a*), tree 2 in 2014 ($N = 5$; *b*), and tree 6 in 2014 ($N = 6$; *c*) were individually challenged against sympatric hosts across 4 years. Time-shift outcomes for the major host genus or genera (>20% of susceptible hosts) were analyzed and plotted here (dashed lines) alongside outcomes for the whole bacterial community (solid lines). The *x*-axes denote the contemporary phage year; the *y*-axes denote the proportion of infected hosts. For readability, *y*-axes show only the range of 0.0–0.4. Average outcomes for pairwise interactions are represented by data points, with the contemporary pairing demarcated by the largest data point. Error bars represent the standard error calculated with the Jeffreys interval for extreme probabilities ($\mathrm{SE} = (\widehat{p}[1-\widehat{p}]/[n+1])^{1/2}$, where $\widehat{p} = (x + 1/2)/(n + 1)$, x is the number of successes, and n is the number of trials). Phages from tree 1 in 2013 (*a*) are more infective on past *Pseudomonas*, consistent with arms race dynamics, but are much less infective on the whole community from the far past, likely because no *Pseudomonas* were collected from that community, which is in line with broader expectations of fluctuating selection. Phages from tree 2 in 2014 on *Pantoea* (*b*) and phages from tree 6 in 2014 on *Pseudomonas* (*c*) are less infective on far-past hosts, consistent with fluctuating selection dynamics and the time-shift outcomes from the bulk 2014 phage.

(fig. 5*a*), infectivity depended on host time shift (host time shift: estimate $= -1.66$, $\mathrm{SE} = 0.138$, $z = -12.11$, $P \ll .0001$). Using a Tukey's post hoc test, we found that infectivity was higher on past *Pseudomonas* than on contemporary or future hosts (table S3), suggesting ARD. However, it is important to note that there were no *Pseudomonas* hosts collected from tree 1 in 2011. For the phages from tree 2 in 2014 on *Pantoea* (fig. 5*b*), we found a significant effect of host time shift on infectivity (host time shift: estimate $= 0.23$, $\mathrm{SE} = 0.092$, $z = 2.53$, $P = .011$). Using a Tukey's post hoc test, we found that infectivity decreased against far-past hosts, suggesting FSD (table S3). For the phages from

tree 6 in 2014 on both *Erwinia* and *Pseudomonas* (fig. 5*c*), we found no significant effect of host time shift on infectivity ($P = .44$) but found a marginal interaction between host time shift and genus (host time shift × genus: estimate = 0.36, SE = 0.189, $z = 1.93$, $P = .053$). We found no effect of host time shift on infectivity of *Erwinia* ($P = .33$) but found a marginal effect on *Pseudomonas* (host time shift: estimate = 0.26, SE = 0.146, $z = 1.78$, $P = .08$). Using a Tukey's post hoc test, we found that infectivity drops against hosts from the far past relative to the recent past, suggesting FSD (table S3). Data underlying figures 2–5 have been deposited in the Dryad Digital Repository (https://doi.org/10.6078/D1141B; Dewald-Wang et al. 2021).

Discussion

Previous results from time-shift experiments performed within a single growing season in the horse chestnut phyllosphere suggested that, as predicted by theory, both bacterial hosts (Koskella 2014) and lytic phages (Koskella and Parr 2015) performed better (i.e., higher resistance/greater infectivity) against antagonists from earlier in the season than from the contemporary month. The data suggest that phage-mediated selection in this system leads to increased bacterial resistance, either through mutational change or through selection on standing variation at the population or community level, and that resistance selects for counteradaptations in phages. However, whereas phages were observed to be less infective to hosts from much earlier in the growing season and more infective to those from the recent past, hosts were observed to be highly resistant to all phages from previous time points in the season, even against inocula that were highly infective to their own contemporary hosts (Koskella and Parr 2015). This apparent asymmetry in the durability, or range, of infectivity versus resistance suggests an interesting lack of fit for the system to either ARD or FSD, but it could also reflect different efficacies of selection and/or timescales of response to selection across bacteria and phages. Importantly, given the differences in generation times and likely asymmetry in the strength of selection acting on bacteria-phage interactions, we wondered whether the bacterial dynamics observed were simply reflecting too short of an observational window to detect fluctuating selection rather than a true asymmetry in dynamics (fig. 1). Here, we performed time-shift experiments across 4 years and observed a similar asymmetry, where phages were found to be less infective to hosts from previous years, especially those from further in the past, whereas hosts were on average more resistant to all past phages (fig. 3). We examined these patterns at the level of the metacommunity but also focused on patterns observed for the dominant bacterial genera and for individual phages that were isolated and amplified on their sympatric hosts.

The surprising observation that bacterial hosts in the natural phyllosphere environment are generally more resistant against phages from months earlier in the season (Koskella and Parr 2015) as well as from previous years, as shown here, calls into question the idea that phage resistance is lost over time under relaxed selection (e.g., Weissman et al. 2018). There exists evidence from across systems that phage resistance can carry significant fitness costs to the bacterial host (Lennon et al. 2007; Scanlan et al. 2015*a*; Vale et al. 2015) and that these costs can be exacerbated in natural settings, including the plant environment (Meaden et al. 2015). As such, there is good reason to predict that phage resistance should be lost over time if the particular phage-mediated selection is relaxed. The observation that phage resistance is, at least at this coarse-grained scale, durable over time could indicate that the evolved mechanisms are broadly effective against many phages, and thus unlike under models incorporating tight specificity, phage-mediated selection is in fact not relaxed at this scale. This idea is in line with expectations from ARD models but again suggests that these broad resistances are not so costly that they are typically lost through time. Recent experimental evolution work from Betts et al. (2018) found that increasing the diversity of phage in inocula led to a switch from FSD to ARD in host resistance that could help explain our results, given the higher diversity of phages in these natural environments—and likely in our inocula—relative to most in vitro studies. A different pattern may well emerge in cases where evolutionary changes within individual bacterial and phage lineages are measured over time rather than average community-wide dynamics. However, our attempt to look at a finer scale by examining individual bacterial genera and individual phage isolates fell more or less in line with the full community results.

In contrast to the durable host resistance observed, our results suggest that phages lose the ability to infect previous host types across years, in parallel with previous results (Koskella 2014), albeit with far greater variability, as might be expected given the loss of infectivity observed within a single season. There are many possible explanations for this observed pattern, including constraints on adaptation to new host types or novel resistances that lead to loss of previous adaptations (Belshaw et al. 2008). One interpretation could simply be that community turnover made it so that phages isolated in one year were unlikely to encounter the same host taxa in other years. In other words, the host that the phage is specialized on is less likely to be present at noncontemporary time points. However, our analyses focusing on patterns within particular bacterial genera suggest that this is unlikely to be the primary explanation (fig. 4). Moreover, the evidence from isolated individual phages (fig. 5) suggests that these same dynamics can occur

even when examining infectivity between a single phage and bacterial genus (although note that community turnover could still be occurring at the species or strain level). Although it is likely that the observed asymmetry is due in part to reduced phage densities associated with time in the fridge (an unavoidable limitation of these types of time shifts over longer time frames), the results mirror what was observed within a single growing season, where we could rule out the reduced infectivity of phages from months earlier because of their similar infectivity to contemporary hosts (Koskella and Parr 2015). Moreover, the results were robust to exclusion of data from 2011, where phages seemed to be particularly absent and hosts particularly resistant, even against contemporary antagonists. In addition, the 2011 phages were more infective against future hosts, which is consistent with expectations for FSD (Gandon et al. 2008) and suggests that phages collected in 2011 were not so degraded from freezer storage as to render them noninfective.

The large changes in relative abundances of bacterial taxa across communities (both spatially and temporally; fig. 2) could suggest that each leaf is acting as an independent community that is relatively unlinked to either other leaves on the tree or leaves from the same tree but different years. However, from the perspective of bacteria-phage dynamics, this does not seem to be the case. Previous work has shown that phages collected from horse chestnut leaves are similarly infective to bacteria from the same leaf as to those from other leaves on the same tree while being relatively noninfective to bacteria from other trees (Koskella et al. 2011). Follow-up work suggests that phages are even more infective to sympatric bacterial hosts from a month earlier than to either sympatric bacteria from the same month or bacteria from a month earlier but from a different tree (Koskella 2014), suggesting that coevolution is more likely occurring at the scale of the tree than of the leaf. The data presented here build on this by suggesting that phage infectivity on bacterial communities can be linked even across years, wherein phages are more infective on bacteria from recent years and bacteria are more resistant to phages from earlier years, although we did not test allopatric bacteria and phages in this study. If each leaf were acting as an independently coevolving community that is disassembled at the end of a season as leaves fall, we would predict instead that phage infectivity and bacterial resistance should be highest for contemporary time points. How bacterial and phage communities are linked across time, however, remains an open question. It could be the case, for example, that these communities overwinter in the soil and reinoculate the same tree from which they fell, or it could be that these bacteria and phages reside within other structures of the tree, such as phloem or stems, and thus reinoculate leaves upon emergence. Finally, it could be the case that the dynamics are shaped by the tree itself, for example, as a result of defensive chemistry or leaf exudate profiles.

The finding that *Pseudomonas* isolates were generally more resistant to inocula from previous years, whereas *Pantoea* and *Erwinia* were generally equally resistant to inocula from each year, opens questions as to the role of community and population turnover between growing seasons in mediating coevolution between bacteria and phages. Leaf senescence and below-freezing temperatures could potentially influence both interannual variation in phyllopshere community composition and bacteria-phage coevolution, though current evidence of this is scarce. Over much shorter time scales, rapid temporal environmental variation was found to decelerate ARD by inhibiting selective sweeps, while longer-term variation promoted ARD between bacteria and phages (Harrison et al. 2013). However, it is unclear whether more gradual variation could drive ARD over multiple years. One possible explanation for the differences in dynamics observed among taxa is that *Pseudomonas* populations overwinter in/on the tree and are thus contiguous over time, whereas other species reestablish in new leaves each season, thus disrupting the coevolutionary process. Previous work on magnolia tree phyllosphere communities indeed suggests that microbial communities are mostly reset each season but that some continuity seems to occur (Jackson and Denney 2011). Indeed, more recent work on poplar trees suggests clear differences in microbial community composition across microhabitats in the phyllosphere (Cregger et al. 2018). Alternatively, it is possible that bacterial taxa differ substantially in their relative sensitivities to phages overall, for example, as a result of life history trade-offs. Additionally, winter is a potential bottleneck event, which have been shown to influence bacteria-phage coevolution, often in favor of the host, by purging low-frequency susceptible genotypes (Hesse and Buckling 2016; although see Wein and Dagan 2019 for the competing effects of moderate temperature changes) or through a dilution effect in which the resulting smaller populations are less likely to encounter their specific phages (Dennehy et al. 2007; Common and Westra 2019). In *Pseudomonas*, stronger population bottlenecks have resulted in greater and more equal resistance to both sympatric and allopatric phages (Hesse and Buckling 2016), consistent with spatial predictions for ARD (Gandon et al. 2008), and this is perhaps a driver of the durable resistance in *Pseudomonas* observed here.

In addition to the bulked phage inocula, the phages that were isolated and amplified on a single host were also generally more infective against congeneric hosts from the recent past (fig. 5). When looking only at *Pseudomonas*, phages from tree 1 in 2013 tended to be more infective on past hosts and less infective on future hosts, consistent with ARD. However, when looking at the entire sympatric

community, infectivity declines dramatically on hosts from 2011, most likely because no sympatric *Pseudomonas* were collected in 2011. This wider community pattern is in line with expectations of FSD, such that phages were most infective on the novel and naive *Pseudomonas* and were less infective against later *Pseudomonas*. The isolated phage group from tree 2 in 2014 was least infective against their *Pantoea* hosts from 3 years in the past and were most infective against hosts from the recent past and contemporary time points, consistent with FSD. Interestingly, while the pattern in infectivity for the group from tree 2 in 2014 is remarkably similar to that of the bulked 2014 phage inocula, the phage group from tree 1 in 2013 peaked against recent-past hosts, whereas the 2013 bulked phage inocula peaked against contemporary hosts.

Since we performed our time-shift experiments on environmental samples, and not on experimentally coevolved host and phage lineages, it is very possible that our results reflect ecological signatures of phage-bacteria interactions, given the striking temporal and spatial variation in the phyllosphere found in this system, rather than evolutionary trajectories within populations. As such, our experimental results may more accurately measure the asymmetry in range of resistance and infectivity between phages and bacteria and the durability of resistance and infectivity over time rather than evolutionary changes in resistance and infectivity within individual lineages. This contrasts to how time shifts are generally used in measuring coevolution in an experimental evolution design. Therefore, the predictions we tested here differ from those in more traditional time-shift experiments on isolated lineages. One advantage of running these experiments in naturally diverse communities, however, is the potential to capture dynamics resulting from diffuse coevolution (Weitz et al. 2013). Since phage host range is often observed to be multiple strains, if not species or genera (Koskella and Meaden 2013), studying dynamics at the pairwise interaction level could miss critical dynamics.

Although our experiment is not set up to directly measure evolutionary change and disentangle the effects of evolution and ecology on resistance and infectivity over time, we do provide insight into these potentially opposing forces. We found a dissimilarity between infectivity across the phage metacommunity (fig. 3*b*) and within isolated phages on *Pseudomonas* (fig. 5*a*), which is particularly striking given the apparent role of the preferred host's absence in driving community-scale FSD. These differing trajectories in phage-bacteria interactions over time may ultimately result in differential effects when looking at population- versus community-scale dynamics. While our asymmetric results at the metacommunity and metapopulation scales suggest that both ecological and evolutionary processes influence the durability of infectivity and resistance, whether and how these processes may interact in this system remains a critical open question.

Explaining the observed asymmetry remains a challenge given that the theoretical work to date has focused on the dynamics of a given system being symmetrical, typically as a result of the underlying infection genetics assumed. However, multiple possible interpretations exist, including the idea that coevolutionary dynamics in this system are defined primarily by phages adapting with a high level of specificity to common hosts at the cost of being able to infect hosts that they could before this adaptation. This idea is related to that of constrained viral evolution due to smaller genome size (Belshaw et al. 2008). It is also possible that hosts from all but contemporary time points are resistant not by selection but rather by chance. In this case "nonhost resistance" is actually just a byproduct of phage evolution, as the populations adapt to specifically infect the dominant bacterial host in the community at a given time (Antonovics et al. 2013). Along the same lines, the results could indicate an asymmetry in genetic barriers during the arms race, as observed in *Prochlorococcus* and its T7-like phages, where some host resistances select for phage host switching rather than counteradaptation (Schwartz and Lindell 2017). Similar asymmetrical bacterial resistance range and phage infectivity range have also been observed in a marine cyanobacterium (Larsen et al. 2019) and been uncovered in bacteria with a CRISPR-Cas9 system, suggesting that the CRISPR bacterial hosts could maintain resistance to multiple phage variations at little cost, while phages lost infectivity to past host variations because of limitations in genome size and mutation supply (Common et al. 2019; but see Holguín et al. 2019). Furthermore, several studies have suggested or shown asymmetry in the evolutionary potential of bacteria and phages, resulting in the bacteria "winning" the coevolutionary race, as a result of genetic and structural constraints (Lenski 1984; Lenski and Levin 1985; Bohannan and Lenski 2002; Scanlan et al. 2015*b*; Schwartz and Lindell 2017). Together with the previous results from within a season and these data from other systems, these new results suggest that coevolutionary dynamics in natural populations/communities are unlikely to fit the predictions of existing models but, more importantly, can be measured using the same types of approaches that have proven so powerful in the laboratory. Future studies from diverse systems will be required before we can determine how widespread asymmetries in coevolutionary dynamics among hosts and parasites might be, and if indeed these patterns are common, there will be need to revise existing theory to determine whether and how such asymmetries change our predictions regarding the impact of coevolution on diversity, local adaptation, and molecular evolution.

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Statement of Authorship

This work was conceptualized and funding was secured by B.K., and the methods were developed by B.K., N.P., and K.T. Data were collected by N.P. and K.T. and analyzed by E.A.D.-W. and A.L. The manuscript was written by E.A.D.-W. and B.K., with feedback from N.P., K.T., and A.L.

Data and Code Availability

All data from experimental crosses in this article have been deposited in the Dryad Digital Repository (https://doi.org/10.6078/D1141B; Dewald-Wang 2021). All accessible sequence data have been deposited in NCBI GenBank (accession numbers: MW632238–MW632724). R code is provided in a zip file, available online.[1]

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1. Code that appears in *The American Naturalist* is provided as a convenience to readers. It has not necessarily been tested as part of peer review.

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"Looking now at our cross-grain shaving of oak, we notice first, scattered somewhat unevenly all over it, large openings, which are the spiral ducts; in some parts they appear to be more closely congregated together, forming, as it were, rows which are continuous after the manner of rings, increasing in dimensions from the centre of the stick towards the circumference. These show us how the wood grows. . . . Thus will the student of nature find instruction and amusement, knowledge and pastime, even in a shaving of wood cast off from a carpenter's jack-plane." From "Shavings Examined Microscopically" by A. M. Edwards (*The American Naturalist*, 1870, 3:561–568).