ECOLOGY LETTERS

Ecology Letters, (2013) **16**: 39–46 doi: 10.1111/ele.12010

LETTER

Effects of predation on real-time host–parasite coevolutionary dynamics

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Abstract

The impact of community complexity on pairwise coevolutionary dynamics is theoretically dependent on the extent to which species evolve generalised or specialised adaptations to the multiple species they interact with. Here, we show that the bacteria *Pseudomonas fluorescens* diversifies into defence specialists, when coevolved simultaneously with a virus and a predatory protist, as a result of fitness trade-offs between defences against the two enemies. Strong bacteria–virus pairwise coevolution persisted, despite strong protist-imposed selection. However, the arms race dynamic (escalation of host resistance and parasite infectivity ranges) associated with bacteria–virus coevolution broke down to a greater extent in the presence of the protist, presumably through the elevated genetic and demographic costs of increased bacteria resistance ranges. These findings suggest that strong pairwise coevolution can persist even in complex communities, when conflicting selection leads to evolutionary diversification of different defence strategies.

Keywords

Antagonism, arms race dynamics, community ecology, conflicting selection, diffuse coevolution, experimental evolution, fluctuating selection dynamics, *Pseudomonas fluorescens*, *Tetrahymena thermophila*.

Ecology Letters (2013) **16**: 39–46

INTRODUCTION

Coevolution, the reciprocal evolution of interacting species, almost certainly plays a fundamental role in structuring natural communities and the evolution and maintenance of biodiversity in general (Thompson 2005, 2009). Community structure – which determines whether a species interacts with one or many other species – is in turn likely to alter the reciprocal selection pressures between species, and hence the dynamics of coevolution (Fox 1988; Thompson 2005; Johnson & Stinchcombe 2007; Bascompte 2009). Although observational and experimental work suggest that both selection and (co)evolutionary outcomes are affected by the number of interacting species within communities (Iwao & Rausher 1997; Stinchcombe & Rausher 2001; Thompson & Cunningham 2002; Strauss *et al.* 2005; Thompson 2005; Berenbaum & Zangerl 2006; Craig *et al.* 2007; Edeline *et al.* 2008; Parchman & Benkman 2008; Siepielski & Benkman 2008; Gómez *et al.* 2009; Koskella *et al.* 2011), these studies necessarily represent phenotypic snapshots in time, and tell us little about coevolutionary dynamics (Nuismer *et al.* 2007, 2010). This is an important limitation because many implications of coevolution, such as its impact on ecological population dynamics (Yoshida *et al.* 2003; Buckling & Hodgson 2007) and selection for sexual reproduction (Hamilton 1980), are dependent on the details of the dynamics. Here, we take an alternative approach to the above studies to determine how community complexity affects pairwise coevolution: we experimentally coevolve populations of microorganisms in real-time and directly measure coevolutionary dynamics.

Pairwise antagonistic coevolution will be affected by the presence of an additional interacting species if selection pressures imposed upon one of the species by the other two are correlated (Iwao & Rausher 1997; Strauss *et al.* 2005). Where investigated, these correlations typically appear to be negative, with the presence of one enemy reducing selection pressures imposed by another (Iwao & Rausher 1997; Stinchcombe & Rausher 2001; Strauss *et al.* 2005; Thompson 2005; Berenbaum & Zangerl 2006; Craig *et al.* 2007; Edeline *et al.* 2008; Parchman & Benkman 2008; Siepielski & Benkman 2008; Gómez *et al.* 2009; Koskella *et al.* 2011). There are two non-mutually exclusive reasons for this. First, there are sometimes direct or indirect genetic trade-offs between defence against multiple enemies, such that selective benefits of defence against one enemy are reduced by the corresponding reductions in defence against the other (Davies & Brooke 1989; Stinchcombe & Rausher 2001; Thompson & Cunningham 2002; Berenbaum & Zangerl 2006; Nuismer & Thompson 2006; Craig *et al.* 2007; Edeline *et al.* 2008; Gómez *et al.* 2009; Siepielski & Benkman 2010). Note, however, that some macro-coevolutionary patterns have been explained by an absence of such trade-offs: for example, the thickness of invertebrate shells is argued to be a generalised adaptation against multiple predators that impose similar ('diffuse') selection pressures for defence (Vermeij 1994). Second, the addition of another exploiter is likely to reduce the population sizes of both species in a pairwise antagonistic interaction, which in turn will reduce encounter rates and the strength of selection for costly defence (e.g. Hochberg & Baalen 1998; Lopez-Pascua & Buckling 2008).

A lowered response to selection for defence against a single enemy will in turn reduce selection for counter-defence, weakening pairwise coevolution. The extent to which pairwise coevolution is weakened is likely to crucially depend on the evolved defence phenotype of individual host or prey organisms. Multiple enemies may

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result in a phenotype that is relatively weakly defended against all enemies (Stinchcombe & Rausher 2001; Thompson & Cunningham 2002; Berenbaum & Zangerl 2006; Craig *et al.* 2007; Gómez *et al.* 2009). Coevolution will then be 'diffuse', with the focal species evolving in response to the enemy community as a whole. In this case, pairwise coevolution may be reduced or even eliminated. Alternatively, and assuming trade-offs in defence, multiple enemies may result in divergent selection for specialist defence strategies, where different sub-populations are primarily adapted to different interacting species (Futuyma & Moreno 1988; Davies & Brooke 1989; Nuismer & Thompson 2006; Edeline *et al.* 2008; Siepielski & Benkman 2010). Each sub-population may then effectively coevolve independently with their respective enemies either simultaneously or sequentially (the latter termed 'coevolutionary alteration'; Davies & Brooke 1989; Nuismer & Thompson 2006), maintaining stronger pairwise coevolution, at least for some of the time.

In addition to weakening pairwise coevolution, the presence of additional enemies might also qualitatively alter coevolutionary dynamics. At one extreme of a simplified continuum, coevolution is driven by directional selection for hosts and parasites that can resist/infect increasing numbers of parasite/host genotypes (Arms Race Dynamic; ARD) (Agrawal & Lively 2002; Gandon *et al.* 2008). At the other extreme, coevolution is driven by negative frequency dependent selection, with different specialist genotypes dominating at different points in time (Fluctuating Selection Dynamic; FSD) (Agrawal & Lively 2002; Gandon *et al.* 2008). Although coevolutionary dynamics (i.e. ARD or FSD) result to some extent from the underlying genetic specificity between host and parasite (Buckling & Brockhurst 2012), both theory (Sasaki 2000; Agrawal & Lively 2002) and experiments (Gómez & Buckling 2011; Hall *et al.* 2011) suggest that ARD can switch towards FSD if ecological conditions reduce selection for costly resistance. As such, the presence of additional enemies could potentially shift an ARD towards a FSD.

Here, we experimentally determined how pairwise coevolutionary dynamics between a host and a parasite are influenced by the presence of a predator, whether selection due to multiple enemies result in generalised vs. specialised defensive strategies, and if the demographic consequences differ in the presence of single vs. multiple enemies. We studied coevolution in real-time by using a microbial system, where *Pseudomonas fluorescens* prey bacterium was exposed for 4 weeks to an obligately killing virus, a bacteriophage (phage) Φ2, in the presence and absence of a predatory protist (*Tetrahymena thermophila*, Ciliates). It has previously been shown in similar experimental settings that: (1) both enemies can significantly reduce bacterial densities, thus imposing strong selection for defensive adaptations (Meyer & Kassen 2007; Gómez & Buckling 2011); (2) there is extensive reciprocal selection for resistance and infectivity between bacteria and bacteriophage Φ2 over a matter of weeks, primarily characterised by monotonic increases in infectivity and resistance (ARD; Buckling & Rainey 2002; Hall *et al.* 2011); (3) bacteria can rapidly evolve increased defence against *Tetrahymena* protist (Meyer & Kassen 2007); (4) defensive adaptations against both enemies incur growth rate costs (Meyer & Kassen 2007; Gómez & Buckling 2011). The effect of parasites on protist–bacterium coevolution was not considered in this study because *T. thermophila* did not evolve in response to *P. fluoresecens* in previous experiments under similar conditions (Meyer & Kassen 2007). We found that concurrent selection by both enemies led to bacterial diversification into specialist defensive strategists due to a trade-off between defences against phages and protists, and that the presence of protists reduced the densities of both bacteria and phages. Consistent with general theory, the pairwise ARD between bacteria and phages was weakened in the presence of protist, with data also consistent with more of a shift towards FSD. That said, strong pairwise coevolutionary dynamics between bacteria and phages were retained in both the absence and presence of protists, which is consistent with selection favouring specialists defenders that interact primarily with a single enemy species (Nuismer & Thompson 2006).

MATERIALS AND METHODS

Strains, selection experiment and species' population density measurements

Twenty 25 mL glass vials (microcosms), each containing 6 mL of 10% King's Medium B (KB; M9 salt solution supplemented with 10 g L^{-1} glycerol and 20 g L^{-1} proteose peptone), were inoculated with 10^6 cells of *P. fluorescens* isolate SBW25 (Buckling & Rainey 2002). Five of the microcosms were inoculated with 4.8×10^5 clonal particles of SBW25Φ2 phage, another five with 4300 cells of *T. thermophila* protist (CCAP strain 1630/1U) and the last five with the same number of both enemies. The microcosms were propagated at 28 °C in non-shaken conditions. One millilitre of each culture was transferred to fresh medium every 7th day, for four transfers. Microcosms were vortexed for 1 min prior to sampling and transfer to fresh media in order to homogenise cultures. Each selection line was maintained for 4 weeks and populations were frozen at −80 °C in 20% glycerol at each sampled time point.

Bacterial densities were determined by plating bacterial dilutions onto KB agar plates and counting the number of colony forming units after 24 h incubation at 28 °C. To isolate phages from bacteria, 100 μL of chloroform was first added to 900 μL of sub-samples, vortexed to lyse the bacterial cells, and then centrifuged at 11 000 ***g*** for 3 min to pellet the bacteria debris, leaving a suspension of phage in the supernatant. Phage population densities were measured by plating phage dilutions onto soft agar plates containing the ancestral bacterial cells and counting the number of plaque forming units after 24 h culture at 28 °C. This method will underestimate phage densities if evolved phages are unable to infect ancestral bacteria. However, majority (> 95%) of phages retain the ability to infect ancestral phages in this system (Hall *et al.* 2011). Protist cells were counted directly under microscope (Leica DM IL Led, 10 × magnification) as described previously (Friman *et al.* 2008).

Bacterial resistance and phage infectivity assays

To measure changes in bacterial resistance and phage infectivity over time, we isolated 16 independent bacterial clones from every population at every time point. Bacterial clones were isolated by picking 16 randomly selected colonies into liquid 10% KB medium for incubation overnight at 28 °C, and freezing in 20% v : v glycerol. Similarly, phage populations were isolated from bacteria with chloroform treatment as described above. Bacterial resistance and phage infectivity were measured at the population level. Resistance of a bacterial population was determined by streaking 16 independent bacterial colonies across a line of phage (40 μL) that had previously streaked and dried on a KB agar plate. A colony was scored as resistant if there was no inhibition of growth by the phage. Bac-

terial resistance was measured as the proportion of resistant bacteria of the 16 colonies (Buckling & Rainey 2002).

We used 'time-shift' assays to measure bacteria–phage coevolution (Buckling & Rainey 2002; Brockhurst *et al.* 2003). Specifically, we estimated the resistance of bacterial populations at every time point to sympatric phages isolated from current or from one time point in the past or future (Brockhurst *et al.* 2003; Lopez-Pascua & Buckling 2008; Gómez & Buckling 2011), allowing resistance and infectivity evolution of bacteria and phage to be determined. To further quantify resistance and infectivity ranges, we also measured bacterial resistance against contemporary, allopatric phage populations by randomly pairing bacterial populations with phage populations between phage–bacteria and phage–bacteria–protist treatments. Bacterial resistance against ancestral phage was measured at weeks 1 and 4.

Measuring bacterial defence against protists

The same bacterial clones that were used for phage-resistance assays at week 4 were used to determine bacterial defence against ancestral protist predators at the end of experiment. The 16 independent bacterial clones per population were first grown to similar densities on 96-well plates [24 h, 28 °C and in 200 μL of 10% KB medium; no differences between treatments: $F_{4,20.2} = 1$, $P = 0.4$, Biotek, OD 600 nm (Bio-Tek Instruments, Inc., Winooski, VT, USA)] before adding 30 μL of protist inocula (1000 cells mL^{-1}). After 24 h of co-cultivation at 28 °C, protist cell numbers were counted under microscope (Leica DM IL Led, 10 × magnification). Bacterial defence was indirectly determined from the number of protist cells: the fewer protist cells, the higher the bacterial defence. Protist cell numbers were converted into 'bacterial defence' by subtracting the cell numbers of experimental treatments from the cell numbers of ancestral bacteria.

Measuring bacterial growth

To determine if defences against different enemies was costly in terms of reduced growth, bacterial maximum growth rates and maximum densities were measured in the absence of enemies at week 4 (Biotek; OD 600 nm, 24 h, 28 °C and in 200 μL of 10% KB medium). The same bacterial clone isolates that were used for phage-resistance and defence against protists were also used for these growth measurements.

Statistical analyses

All time-structured data were analysed as general linear mixed models (GLMM) with unstructured covariance structure, where community composition was used as a categorical explanatory variable, time (week) as a continuous variable and selection line (nested within community) as a random factor. GLMMs were also used for non-time structured data, with community composition as a categorical explanatory variable and selection line as a random factor. Bacterial clones were nested within populations and populations within treatments. Bacterial and phage population density data were log-transformed and all proportional data arcsine-transformed before analyses. Correlation coefficients (Pearson's r) for bacterial resistance and phage infectivity against time (counterpart from past, current and future time point per week) were calculated at each time point for: (1) all replicates combined within a treatment; and (2) for each replicate separately.

RESULTS

Population dynamics

Protist predation caused large reductions in bacterial and phage densities in both single- and two-enemy communities ($F_{1,16} = 36.2$, $P < 0.001$ and $F_{1,8} = 81.9$, $P < 0.001$, respectively, Fig. 1a–b). Phages had no effect on bacterial and protist densities under this sampling regime in this relatively low nutrient media ($F_{1,16} = 0.67$, $P = 0.42$ and $F_{1,8} = 0.07$, $P = 0.79$, respectively, Fig. 1a and c). Protist predation did not directly affect phage densities when cultured in the absence of bacteria ($F_{1,8} = 3.6$, $P = 0.1$, Figure S1). Although protist densities decreased through time (time: $F_{3,32} = 3.5$, $P = 0.02$, Fig. 1c), phage densities tended to increase through time only in two-enemy communities, even though protists drove phage extinct in one of the replicate populations at week 4 (time × community: $F_{3,6.9} = 18.4$, $P = 0.001$; effect of time significant when extinct phage population excluded from the analysis: $F_{3,7.5} = 159.6$, $P < 0.001$, but non-significant when all populations included: $F_{3,4} = 1.3$, $P = 0.4$; light grey lines depict replicate phage populations in Fig. 1b). Time had no effect on phage densities in the absence of protist ($F_{3,4} = 1.2$, $P = 0.4$). This change in the relative density of different enemies resulted in an increase in phage-to-bacteria and decrease in protist-to-bacteria ratio in two-enemy communities (enemy type: $F_{1,8} = 12$, $P = 0.008$, Fig. 1d).

Evolution of bacterial defence against protist and phage enemies

Bacteria evolved in the absence of enemies showed no increase in defence against protists (community: $F_{4,19.3} = 6.7$, $P = 0.001$, $P = 0.5$ for pair wise comparison with ancestral strain, Fig. 2a) or ancestral phage (community: $F_{4,19} = 5.5$, $P = 0.004$, $P = 0.3$ for pair wise comparison with ancestral strain, Fig. 2b). Phage selection in single-enemy communities resulted in moderate cross-resistance against ancestral protist ($P < 0.001$ for pair wise comparison with bacteria-only community, Fig. 2a), while protist selection did not confer cross-resistance to ancestral phage ($P > 0.2$ for pair wise comparison with bacteria-only community, Fig. 2b). Most importantly, bacterial defence against ancestral protist and phage evolved to be greatest in the single-enemy communities demonstrating that responses to selection differed when one vs. two enemies were present (defence against ancestral protist: $P < 0.003$ when comparing protist-only community with all treatments, Fig. 2a; resistance against ancestral phage: $P < 0.01$ when comparing phage-only community with other treatments, Fig. 2b).

We next concentrated on populations exposed to both enemies and investigated if individual bacterial clones had evolved general resistance that was effective against both enemies, or if populations had diversified into specialist resistance strategies. By using defence data from the end of the experiment (week 4), we found that bacterial clones that were resistant to sympatric phages were less defended against protists, whereas bacterial clones that were susceptible to contemporary sympatric phages were better defended against protists (phage resistance × community: $F_{1,144} = 5.24$, $P = 0.023$; phage resistance within bacteria–phage–protist community: $F_{1,70} = 8.7$, $P = 0.004$, Fig. 2c). Bacteria were equally defensive

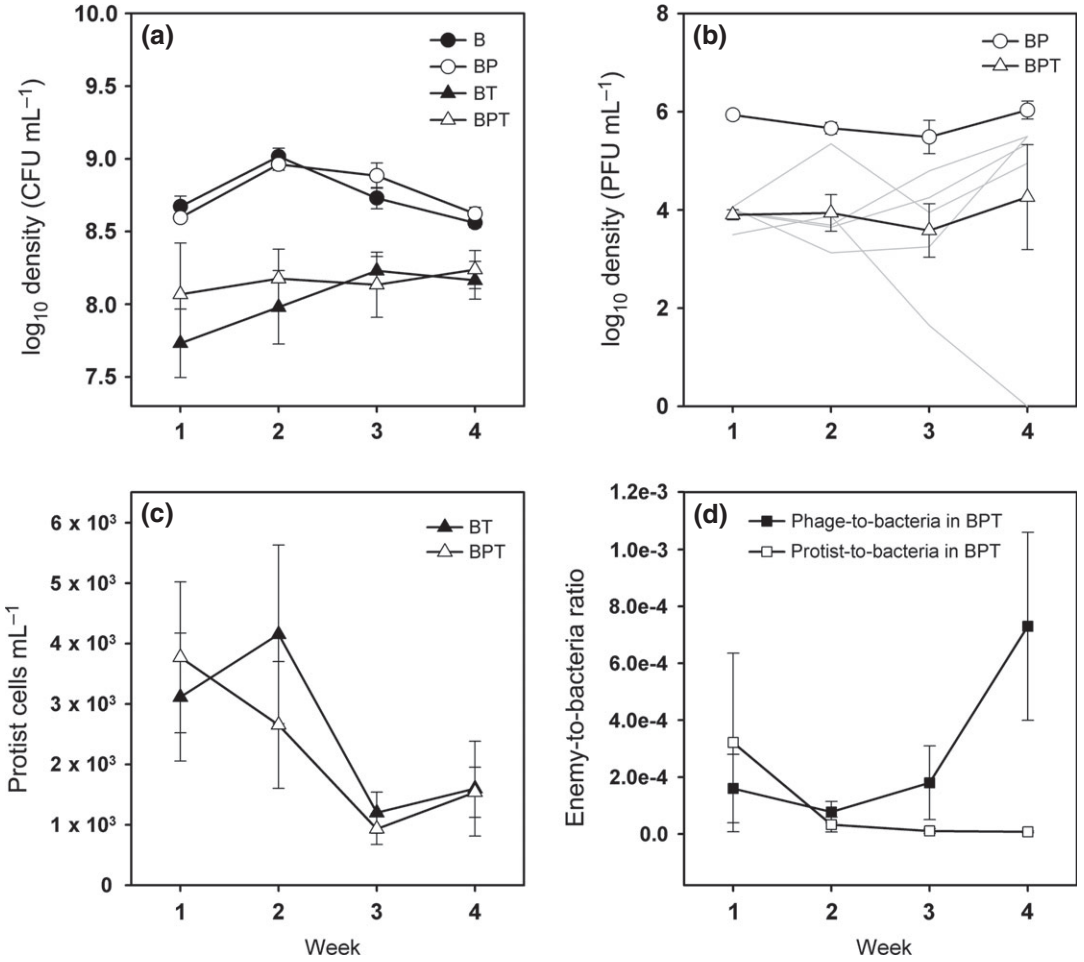

Figure 1 The population dynamics of bacteria, phages and protists, and enemy-bacteria ratios in two-enemy community. (a) Bacterial densities when evolving alone (filled circles, B), in the presence of phage (open circles, BP), in the presence of protist (filled triangles, BT) or in the presence of both phage and protists (open triangles, BPT). (b) Phage densities in the absence (open circles, BP) and presence of protists (open triangles, BPT). Grey lines in panel b depict replicate populations in BPT treatment. (c) Protist densities in the absence (filled triangles, BT) and presence of phage (open triangles, BPT). (d) Phage-to-bacteria (plaque-to-colony) and protist-to-bacteria (cell-to-colony) ratios in two-enemy communities. In all panels, $n = 5$ and for all data points and error bars denote ± 1 SEM.

against protists regardless of their resistance to phages within the phage-only treatment (phage resistance: $F_{1,71} = 0.6$, $P = 0.42$, Fig. 2c). Similarly, bacterial defence against protists did not correlate with resistance to ancestral phage within the protist–bacteria treatment (phage-resistance: $F_{1,63} = 2.4$, $P = 0.12$, data not shown). These results suggest that concurrent selection by both phage and protist diversified bacterial populations into specialist resistance strategies in two-enemy communities.

Costs of defences in the absence of enemies

To determine costs associated with defence, we measured both the maximum densities and maximum growth rates achieved by bacterial populations in the absence of enemies. Only the populations that were exposed to both enemies suffered a reduction in bacterial maximum growth rates (community: $F_{3,16} = 3.2$, $P = 0.05$, $P < 0.004$ in all multiple comparisons, Fig. 2d). Similarly, while bacterial maximum densities decreased due to protist-selection in both the presence and absence of phages, this decrease was greatest in the presence of phages (community, $F_{3,16} = 6.5$, $P = 0.004$, $P < 0.001$ in multiple comparisons, $P = 0.01$ for single vs. two-enemy communities, Fig. 2d); phage selection alone did not result in any apparent growth cost ($P > 0.05$ in all comparisons). These data suggest that defence against protists and phages impose some competitive costs, with protist defence imposing the greater cost.

The effect of protist on phage–bacteria coevolutionary dynamics

Each week, the resistance of bacteria was determined against their contemporary co-occurring phages, as well as phages (isolated from the same evolving communities) from a week in the past and week in the future. Bacteria and phage underwent short-term arms race coevolutionary dynamics (ARD) in both the absence and presence of protist; bacteria evolved increased resistance and phages evolved increased infectivity through time (past, contemporary and future populations) with respect to their contemporary counterpart (bacterial resistance: $F_{2,15} = 154.7$, $P < 0.001$; phage infectivity: $F_{2,18.6} = 97$, $P < 0.001$; interactions with bacterial resistance and community: $F_{2,15.04} = 5.7$, $P = 0.014$ and phage infectivity and community: $F_{2,18.6} = 3.1$, $P = 0.064$, Fig. 3a–b). The mean rate of ARD was not significantly reduced by the presence of protist (community:

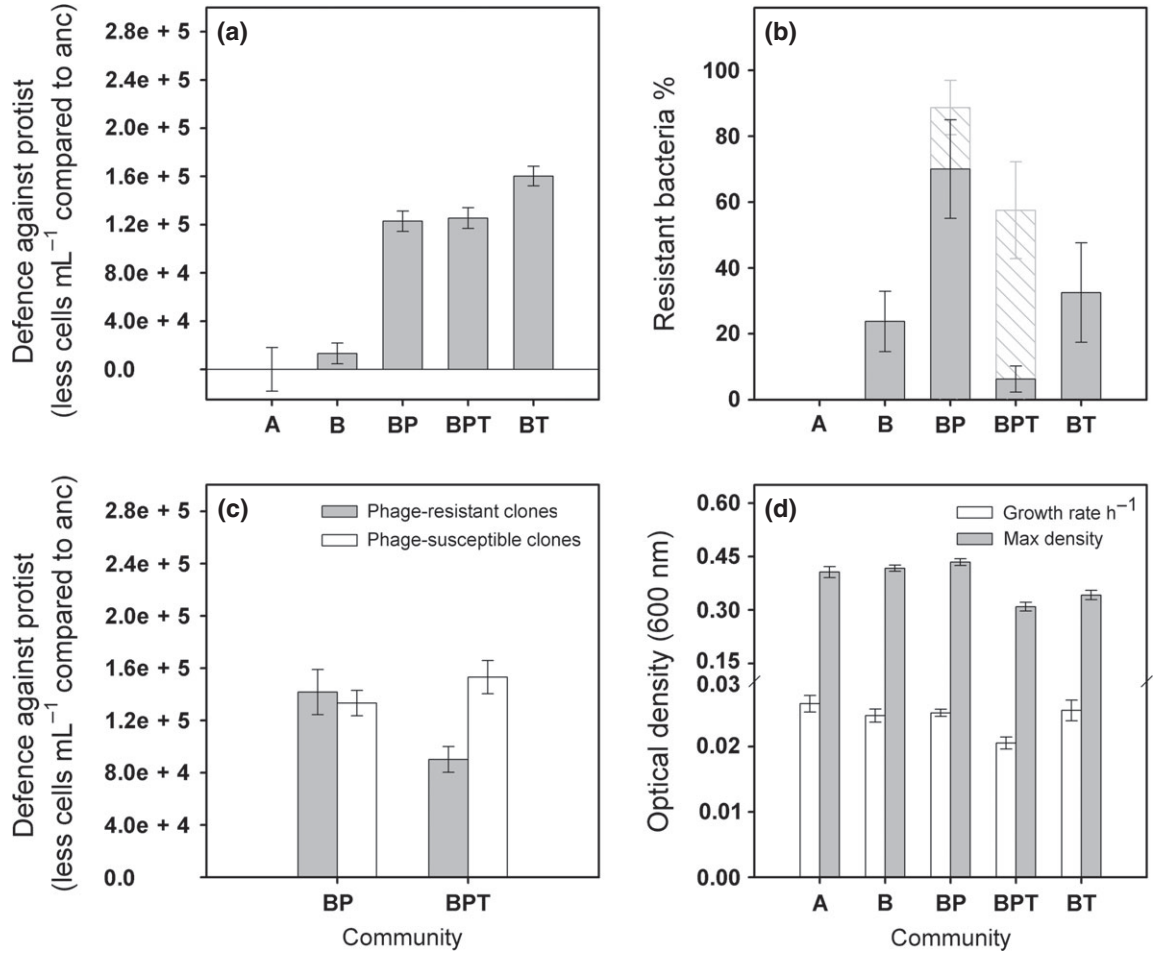

Figure 2 (a) Bacterial defence against ancestral protists at week 4 when bacteria had evolved alone (B), in the presence of phage (BP), protist (BT) or both enemies (BPT), compared with ancestor bacteria (A). (b) Bacterial resistance to ancestral phage at week 4 when bacteria had evolved alone (B), in the presence of phage (BP), protist (BT) or both enemies (BPT), compared with ancestor bacteria (A). Light grey, shaded bars in panel b show resistance against sympatric, contemporary phages. (c) Phage-resistant and phage-susceptible clones' defence against ancestral protists at week 4. (d) Bacterial maximum growth rate (white bars) and maximum density (grey bars) in the absence of enemies, when bacteria had evolved for 4 weeks alone (B), in the presence of phage (BP), protist (BT) or both enemies (BPT), compared with ancestor bacteria (A). In all panels, $n = 5$ for all communities and error bars denote ± 1 SEM.

$F_{1,8} = 4.1$, $P = 0.08$; defined as mean change in resistance to future phage minus resistance to phage from past).

Although the mean rate of ARD was similar, other aspects of the coevolutionary dynamics differed considerably between the two treatments (Fig. 3a–b). First, while bacteria had evolved almost complete resistance to the ancestral phage after week 1 in both treatments (Fig. 3a–b), bacteria had almost entirely lost resistance against ancestral phage by week 4 when coevolving in the presence of protist, whereas resistance loss to ancestral phage was much less when evolving in the absence of protist (Fig. 2b). Second, the arms race dynamic characterised by an increase in resistance and infectivity through time appeared to break down at latter time points in the presence of protist (Fig. 3a–d). Specifically, treatment-level bivariate correlations of bacterial resistance and phage infectivity against time were only significant in the first week in the presence of protists, whereas they were retained in the absence of protists (total of 2/6 cases significant compared with 5/6 significant cases in the phage-only community, Table S1). Similarly, phage infectivity decreased through time in the presence of protist when comparing means of population level correlation coefficients (community: $F_{1,6.1} = 18.6$, $P = 0.005$, community × time: $F_{2,7.2} = 8$, $P = 0.014$, Figure S2), while in the case of bacterial resistance a significant difference was observed only at week two (community: $F_{1,8.8} = 1.6$, $P = 0.2$, Figure S2). Third, mean resistance against both co-occurring (sympatric) phages (community: $F_{1,8} = 9.7$, $P = 0.014$, Fig. 3c–d) and phages isolated from the same time point but the other treatment (allopatric) ($F_{1,8} = 15$, $P = 0.005$, Fig. 3c–d) evolved to be greater in the absence of protists, demonstrating the evolution of a narrower resistance range. Taken together, these data suggest that the ARD was weakened in the presence of the protist.

DISCUSSION

Here, we experimentally studied how selection by predators and parasites affect the evolutionary diversification of prey defence strategies, and consequently, how pairwise coevolution between the host and the parasite is changed in the presence of the predator. Our results show that bacterial defences against both enemies evolved to be greater in single-enemy communities than when both enemies were present. Instead of generalist defensive strategy concurrent

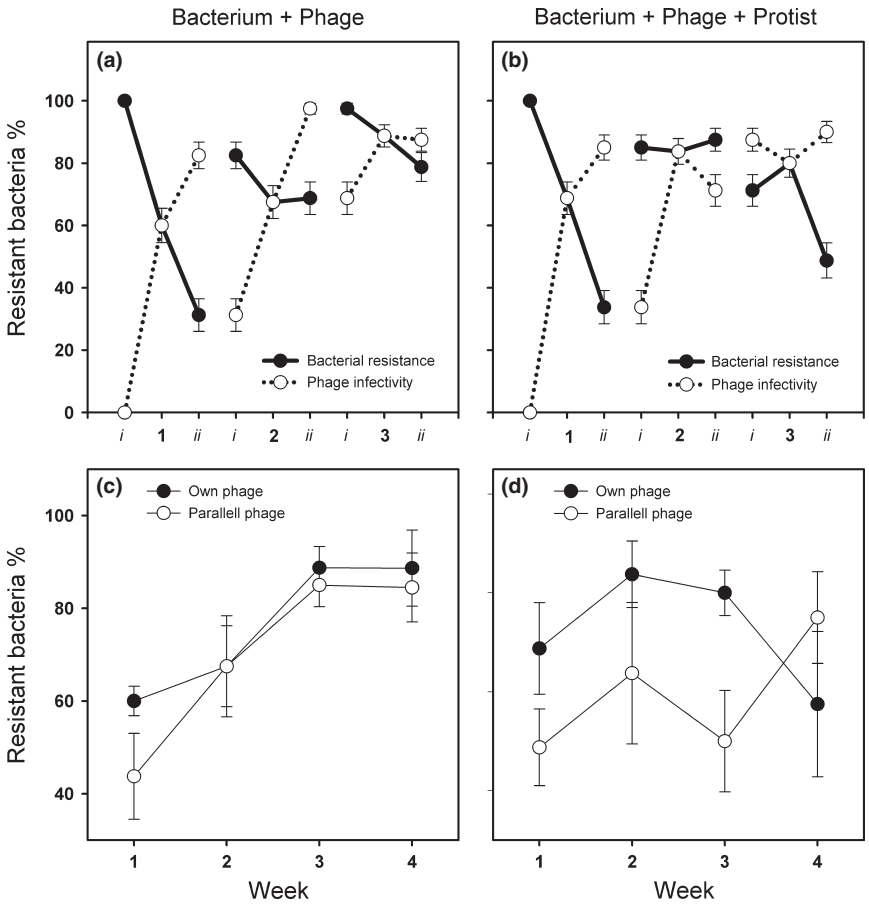

Figure 3 The phage–bacteria coevolutionary dynamics in the absence (a and c) and presence (b and d) of protists. In (a) and (b), solid black lines denote bacterial resistance to contemporary phages (1–3) and resistance to phages from 1 week in the past (*i*) and the future (*ii*). Dotted lines in (a) and (b) denote phage infectivity to contemporary bacteria (1–3) and infectivity to bacteria from 1 week in the past (*ii*) and the future (*i*). (c) The mean bacterial resistance against contemporary phages from own (filled circles) and parallel (empty circles) treatment populations (BPT). (d) The mean bacterial resistance against contemporary phages from own (filled circles) and parallel (empty circles) treatment populations (BP). In all panels, $n = 5$ for all data points and error bars denote ± 1 SEM.

selection by two enemies led to divergence into specialised defenders against predators and parasites. Potentially, as a result of the evolution of specialised defence against phages, bacteria and phage underwent extensive pairwise coevolution in the presence of protists, as well as in their absence; however, coevolutionary dynamics qualitatively differed.

Coevolution initially followed an arms race dynamic commonly observed between these organisms (Buckling & Rainey 2002; Hall *et al.* 2011) in both communities, with both bacteria and phage showing increases in resistance and infectivity ranges through time. However, coevolution was by no means a pure ARD with bacteria rapidly gaining and then subsequently losing resistance to ancestral phages in both communities. Crucially, this loss was much greater in the presence of protist (Fig. 2b). Moreover, protist predation was associated with other indications of a breakdown of ARD: (1) slopes of resistance and infectivity against time showed less monotonic increases, the direct measure of ARD (Fig. 3a and b); and (2) bacterial resistance to phages coevolving in the absence of protist was reduced (Fig. 3c and d); resistance range has been shown to correlate positively with the rate of ARD in previous studies using this system (Brockhurst *et al.* 2003; Lopez-Pascua & Buckling 2010). Both theory (Sasaki 2000; Agrawal & Lively 2002) and data (Gómez & Buckling 2011; Hall *et al.* 2011) suggest that a breakdown of ARD (resulting from elevated costs of resistance, as here) might be accompanied by a shift towards a FSD. However, it is also entirely plausible that strong protist-imposed selection reduced the relative importance of phage-imposed selection, breaking down pairwise coevolution. Without more data, it is impossible to determine if the increased breakdown of ARD in the presence of protists is simply a breakdown of pairwise coevolution or a greater shift towards FSD: unambiguously measuring FSD can be extremely difficult even when coevolutionary dynamics are 'pure' FSD (Gandon *et al.* 2008; Gaba & Ebert 2009), rather than a mix of ARD and FSD (Hall *et al.* 2011). For example, the gain of almost complete resistance to the ancestral phage, followed by the almost complete loss over the 4-week duration of the experiment in the presence of protist is strongly consistent with fluctuating selection on bacterial resistance traits (Gandon *et al.* 2008). However, a similar gain and loss of resistance is not consistently observed over periods of 1 week (Fig. 3a and b). As a result, phenotypic and genetic analyses of much longer term experiments might allow FSD to be unambiguously identified (e.g. Hall *et al.* 2011).

Two important factors may have contributed to the observed reduction in defence evolution to single enemies when both ene-

mies were present: demography and trade-offs. First, protists directly reduced bacterial densities, and indirectly the phage densities. These density reductions likely reduced bacteria–phage encounter rates, and hence, weakened the strength of selection for bacterial resistance against phages and in turn phage infectivity (Hochberg & van Baalen 1988; Brockhurst *et al.* 2003; Lopez-Pascua & Buckling 2008). Moreover, protist-driven reduction in population sizes also likely reduced mutation supply rates (de Visser *et al.* 1999). In contrast, phages did not reduce bacterial densities measured after a week's growth, as previously observed in this relatively low nutrient media (Benmayor *et al.* 2008). That is not to say phages do not influence bacterial densities in this context: they are likely to have increased the time taken to achieve maximal densities, but this was impossible to measure here without destructively sampling from the microcosms. Second, consistent with previous studies, defence against one enemy constrained the defence against the other enemy (Rigby & Jokela 2000; Poitrineau *et al.* 2003; Edeline *et al.* 2008; Friman *et al.* 2009; Siepielski & Benkman 2010): phage-resistant bacterial clones were less defended against protist compared with phage-susceptible clones. Moreover, this trade-off was to some extent indirect, mediated through trade-offs with other fitness-related traits: increased defence against two enemies reduced bacterial growth rate and carrying capacity more than defence against a single enemy. However, it remains unclear to what extent defence to one enemy directly reduced defence to the other.

The evolution of specialised resistance is crucial to the maintenance of pairwise coevolutionary interactions at the community level (Nuismer & Thompson 2006). But why did bacteria diversify in their resistance strategies rather than evolving generalised defence? The evolution of specialist and generalist strategies will be affected by the shape (e.g. convex or concave) of the trade-off curve (Boots & Haraguchi 1999), but unfortunately it was not possible to determine this. From a more ecologically explicit perspective, specialists are most likely to evolve if selection pressures vary in time and, in particular, space (Maynard Smith & Hoekstra 1980), and this is likely to be the case here. First, the static microcosms used in this study are also likely to create spatial heterogeneity in enemy imposed selection. Oxygen concentrations rapidly decrease with distance from the surface (Koza *et al.* 2011), and it has been demonstrated before that *Tetrahymena* is likely to occupy the air-liquid surface of the broth (Meyer & Kassen 2007). In contrast, phages will simply be associated with bacteria, which inhabit the whole of the microcosm. Second, phage impose greater mortality on growing bacterial cells than cells in stationary phase (phages rely on bacterial metabolism for replication; Adams 1959), and hence, phage-imposed selection is likely to be greatest in the hours after transferring communities to fresh media. In contrast, protist-imposed selection is likely to be less dependent on whether cells are in exponential or stationary phase. Finally, across longer time scales, a decline in protist densities towards the end of the experiment was associated with both increased phage densities (Fig. 1b and d) and increased phage infectivity (Fig. 3d). This suggests that the relative strength of selection imposed by different enemies changed through time. Although this finding is broadly consistent with the idea of coevolutionary alternation where species pairs adapt to each other sequentially (Nuismer & Thompson 2006), time-series defence assays against both enemies would be needed to explicitly demonstrate this.

Despite this strong evidence for specialisation of defensive strategies, we found that phage-selection conferred some cross-resistance to protists, whereas protist selection did not increase resistance to phages (Fig. 2a–b). This result is most likely to be explained by increased selection for a mucoid phenotype in the presence of phages. The mucoid phenotype results from over-expression of exopolymers (Scanlan & Buckling 2012) and was to some extent favoured by abiotic experimental conditions: emergence of mucoid bacterial phenotypes was observed in all experimental treatments during the experiment, while their frequency was especially high in the phage-only treatment. Crucially, the mucoid phenotype has been shown to confer partial resistance against phages in this system (Scanlan & Buckling 2012) and is also associated with the formation of cell aggregations (biofilms), which can be inedible for protist predators due to their large size (Meyer & Kassen 2007). The role of correlated selection by abiotic factors for the tolerance of biotic stress (e.g. enemies) will be thus studied more comprehensively in the future.

In summary, we show that strong pairwise coevolutionary interactions occur in more complex communities, but these interactions may be qualitatively altered by third-party interference. Our results may help to explain the often-observed mismatch between phenotypic traits of coevolving species within the same geographical area (Thompson 2005). Specifically, such mismatch is inevitable if coevolutionary pairwise interactions vary in space and time as a result of complex community interactions. Moreover, such variation in the strength of pairwise coevolutionary interactions further suggest that single snapshots in time may tell us little about coevolutionary dynamics (Nuismer *et al.* 2007, 2010): increasing community complexity is likely to be important in creating coevolutionary selection mosaics in both space and time.

ACKNOWLEDGEMENTS

We thank Mike Brockhurst and 5 anonymous referees for helpful comments. This study was funded by Marie-Curie Intra-European Fellowship within the 7th European Community Framework Programme (V.F.). A.B. acknowledges support from the European Research Council.

AUTHORSHIP CONTRIBUTIONS

V.F. designed and performed the experiment and analysed all the data. A.B. provided all the reagents. Study conceived and manuscript written by V.F and A.B.

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Editor, James Grover
Manuscript received 27 June 2012
First decision made 26 July 2012
Manuscript accepted 30 August 2012