The relative importance of evolutionary dynamics depends on the composition of microbial predator-prey community

Ville-Petri Friman (vifriman@gmail.com)[1,2*], Alessandra Dupont (a.dupont@nhm.ac.uk)[3], David Bass (d.bass@nhm.ac.uk)[3], David J. Murrell (d.murrell@ucl.ac.uk)[4] and Thomas Bell (thomas.bell@imperial.ac.uk)[1]

[1]Imperial College London, Silwood Park Campus, Buckhurst Road, Ascot, Berkshire, SL5 7PY, UK

[2]University of York, Department of Biology, Wentworth Way, York, YO10 5DD, London, UK

[3]Natural History Museum London, Department of Life Sciences, Cromwell Road, SW7 5BD, London, UK

[4] University College London, Department of Genetics, WC1E 6BT, London, UK

***Corresponding author:** Ville-Petri Friman; University of York, Department of Biology, Wentworth Way, York, YO10 5DD, London, UK; vifriman@gmail.com; telephone: +44 1904 328675; fax: +44 1904 328505

Statement of authorship: VPF, AD and DB and TB designed the study, AD and VPF performed the research, VPF wrote the first draft of the manuscript and all authors contributed substantially to revisions.

Short running title: eco-evolutionary community dynamics

Subject category: Microbial population and community ecology

Authors declare no conflict of interest

ABSTRACT

Community dynamics are often studied in subsets of pairwise interactions. Scaling pairwise interactions back to the community level is however problematic because one given interaction

might not reflect ecological and evolutionary outcomes of other functionally similar species interactions, or capture the emergent eco-evolutionary dynamics arising only in more complex communities. Here we studied this experimentally by exposing *Pseudomonas fluorescens* SBW25 prey bacterium to four different protist predators (*Tetrahymena pyriformis*, *Tetrahymena vorax*, *Chilomonas paramecium* and *Acanthamoeba polyphaga*) in all possible single-predator, two-predator and four-predator communities for hundreds of prey generations covering both ecological and evolutionary time scales. We found that only *T. pyriformis* selected for prey defence in single-predator communities. While, *T. pyriformis* selection was constrained in the presence of the intraguild predator, *T. vorax*, *T. pyriformis* selection led to evolution of specialised prey defence strategies in the presence of *C. paramecium* or *A. polyphaga*. At the ecological level, adapted prey populations were phenotypically more diverse, less stable and less productive compared to non-adapted prey populations. These results suggest that predator community composition affects the relative importance of ecological and evolutionary processes and can crucially determine when rapid evolution has potential to change the ecological properties of microbial communities.

Keywords: conflicting selection / emergent multiple predator effects / diffuse evolution / community ecology / predation / trade-offs

INTRODUCTION

One of the major goals of ecology is to try to understand the dynamics of complex communities. Traditionally this question has been approached by decomposing food web complexity into more manageable subsets of interacting species, which are then studied in isolation from the rest of the community (Billick and Case, 1994; Vandermeer, 1969). This approach has shown that there are frequently emergent properties that arise only in the presence of multiple species (Sih *et al.*, 1998; Strauss and Irwin, 2004) resulting in ecological and evolutionary outcomes that could not be predicted by on the basis of single- or even two-species dynamics (Berenbaum and Zangerl, 2006; Friman and Buckling, 2014; Friman and Buckling, 2013; Iwao and Rausher, 1997; Parchman and Benkman, 2008; Strauss and Irwin, 2004; Thompson, 2005). We were interested in whether part of the difficulty in predicting multi-species dynamics arises from the feedbacks between ecological and evolutionary processes that are dependent on the precise composition of the predator-prey community.

Recent results have shown that rapid evolution can significantly alter the ecological properties of predator-prey systems. Probably the most convincing evidence comes from microbial predator-prey study systems, where rapid evolution of traits connected to prey defence and predator counter-defence has been observed to change the productivity, stability and diversity of predator-prey communities (Becks *et al.*, 2010; Friman *et al.*, 2008; Friman *et al.*, 2014; Hiltunen and Becks, 2014; Meyer and Kassen, 2007; Yoshida *et al.*, 2003). Even though most of this evidence comes from relatively simple two-species model communities, it has recently been shown that the presence of another predator can affect the temporal dynamics of one-prey-one-predator system (Hiltunen *et al.*, 2013), while modelling work predicts that evolution is more likely to feedback to population dynamics when the prey defence evolves predator-specific (Ellner and Becks, 2011). How predator community complexity affects the outcomes of prey evolution has however not been yet tested experimentally.

Increasing the number of interacting species could affect predator-prey evolution via ecological and genetic constraints. First, competition for the shared prey is likely to affect the

relative abundance of each competing predator species, which will then affect the strength of selection that every predator exerts on the given prey species (Friman and Buckling, 2013). If predator competition is asymmetrical, the most dominant predator species is expected to have strongest effect on prey evolution. If competition between different predators is more symmetrical, both predators are likely to exert selection on prey but these effects are likely to be weaker compared to the effects predators would be exerting on prey in the absence of competition. Second, trait correlations between defence mechanisms against different predators could affect the evolutionary dynamics in multi-predator communities (Friman and Buckling, 2013; Iwao and Rausher, 1997; Strauss and Irwin, 2004; Strauss *et al.*, 2005). In the case of no correlation (independent predator effects), the combined effect of multiple predators may result in divergent selection for specialist defence strategies, where different sub-populations adapt to different interacting species (Davies and Brooke, 1989; Edeline *et al.*, 2008; Futuyma and Moreno, 1988; Nuismer and Thompson, 2006). If defence correlations are negative, selection by one predator could reduce the selection imposed by another predator due to trade-offs in morphology or physiology (Berenbaum and Zangerl, 2006; Davies and Brooke, 1989; Friman and Buckling, 2013; Nuismer and Thompson, 2006; Stinchcombe and Rausher, 2001; Thompson and Cunningham, 2002). It is also possible that defence against one predator correlates positively with the defence against other predator (e.g. due to functional similarity between different enemies). In this case, selection could be 'diffuse' where the prey species evolves in response to the predator community as a whole (Fox, 1988; Thompson, 2005) resulting in a generalist defence phenotype, which is resistant to all predators (Berenbaum and Zangerl, 2006; Craig *et al.*, 2007; Gomez *et al.*, 2009; Stinchcombe and Rausher, 2001; Thompson and Cunningham, 2002).

We used laboratory microbial communities to ask how predator community composition affects the prey evolution and eco-evolutionary dynamics of predator-prey communities. Specifically, *Pseudomonas fluorescens* SBW25, a prey bacterium was exposed to four different bacterivorous protists (*Tetrahymena pyriformis*, *Tetrahymena vorax*, *Chilomonas paramecium* and *Acanthamoeba polyphaga*) in all single-predator, two-predator and four-predator communities for

hundreds of prey generations (for ~ 4 weeks, 24 days); a sufficient timescale to observe changes both in ecological and evolutionary dynamics (Friman and Buckling, 2013; Friman *et al.*, 2014). All selected protist species consumed bacteria and potentially imposed selection for prey defence. Furthermore, *T. vorax* is polymorphic having small microstome and large macrostome morphs (Gronlien *et al.*, 2002). Macrostome morphs are able to feed on other protists (Gronlien *et al.*, 2002) and *T. vorax* could thus potentially affect eco-evolutionary dynamics via intra-guild predation.

We concentrated on both the population and evolutionary dynamics and investigated (i) how prey evolutionary responses depend on the predator species identity in single-predator communities, (ii) whether pairwise predator-prey interactions predict prey evolutionary responses in multi-predator communities, and (iii) whether prey evolution in single vs. multi-predator communities altered the ecological properties of the study system in terms of prey diversity, stability and productivity.

MATERIALS AND METHODS

Study species, culture conditions and selection experiment

We used SBW25 *Pseudomonas fluorescens* as a prey for four protist species (*Tetrahymena pyriformis* ciliate; CCAP #1630/1W, *Tetrahymena vorax* ciliate; CCAP #1630/3C, *Chilomonas paramecium* flagellate; CCAP #977/2A, and *Acanthamoebae polyphaga* amoebae; CCAP #1501/18). The strain SBW25 was originally isolated from a sugar beet leaf (Rainey and Bailey, 1996) and protist cultures were ordered from the Culture Collection for Algae and Protozoa (CCAP). All selected protist species were originally isolated from aquatic environments (Elliott, 1959; Patterson, 1996), were able to feed on the study bacterium, and hence, potentially exerted selection for prey defence (Friman and Buckling, 2014; Friman and Buckling, 2013).

All protists species were cultured axenically in the absence of bacteria before starting the experiment (both *Tetrahymena* ciliates on PPY medium: 20 g L^{-1} peptone and 2.5 g L^{-1} of yeast extract; *C. paramecium* on CHM medium: 1 g L^{-1} Sodium acetate trihydrate and 1 g L^{-1} "Lab-Lemco" powder (Oxoid L29); and *A. polyphaga* on PPG medium: 15 g L^{-1} peptone, 18 g L^{-1} D-

glucose in Page's Amoeba Saline solution (CCAP)). Bacterial stocks were prepared by growing bacteria overnight on LB medium (Sigma-Aldrich; 10 g L^{-1} of tryptone, 5 g L^{-1} of yeast extract and 5 g L^{-1} of NaCl) resulting in final densities of approximately 9×10^7 bacterial cells mL^{-1}.

We used 24-well cell culture plates, each containing 2 mL of 0.5% LB (described above) as microcosms during the selection experiment. The SBW25 bacterium was grown alone and in the presence of all protists in one-, two-, and four-protist species combinations at 22°C in non-shaken conditions. All treatments (twelve in total) were replicated 5 times (N = 5) resulting in total of 60 experimental populations. When initiating the experiment, approximately 2×10^5 bacterial cells mL^{-1} were first added to all populations. All single-predator treatments were subsequently inoculated with ~ 400 protist cells. All two-protist treatments were inoculated with ~ 200 cells per protist species, and four-protist treatment was inoculated with ~ 100 cells per protist species. Microcosms were renewed every fourth day for a total of six times (24 days) by first mixing the contents thoroughly with pipette and then replacing 1 mL of sample with 1 mL of fresh media. Subsamples of all populations were frozen at -80 °C in 20% glycerol at every sampled time point. Rest of the sample was used to define bacterial and protist population densities. Bacterial densities were estimated with Accuri C6 flow cytometer (Becton Dickinson; fast flow rate, 25 µl of sample, a minimum forward scatter threshold of 8000 based on negative controls containing only media). Protist densities were directly counted under the microscope (Motic AE2000, inverted light microscope).

Measuring bacterial defence against protists

Evolutionary changes in bacterial defence against protists were measured at the end of the 24-day long selection experiment. Defence was measured at the level of colony types in order to link bacterial phenotype to certain defence strategy, and to increase measurement accuracy compared to population level measurements. To this end, we randomly isolated 8 independent bacterial colonies per replicate population (50 colonies per treatment; total of 600 colonies), inoculated selected colonies into liquid 0.5% LB medium and incubated overnight at 22°C, and finally, froze the

colonies in 20% glycerol. Even though isolating eight colonies per replicate population might not capture rare colony types, it has been shown to effectively separate defending and non-defending bacterial genotypes within-population level (Friman *et al.*, 2014). Before the defence measurements, all colonies were thawed and grown to similar densities in 96-well plates (24 h, 22°C and in 200 µL of 0.5% LB medium; Biotek, OD 600 nm; mean OD of 0.093 ± 0.001; treatment: $F_{11, 48} = 0.572$, $P = 0.842$). By equilibrating the initial bacterial densities, subsequent protist growth was only affected by differences in the strength of bacterial anti-predatory defence (Friman and Buckling, 2013). Bacterial defence was estimated as the relative fitness in terms of comparing the growth of with-predator-evolved and alone-evolved bacterial selection lines in the presence of ancestral stock predators. To this end, all bacterial selection lines were grown individually with every predator species they had been exposed to during the selection experiment. Briefly, all protist measurement plates were inoculated with 20 µL of ancestral stock protist (approximately 100 cells mL^{-1}) and after 48 h of co-cultivation at 22°C, bacterial defence was determined as the amount of bacterial biofilm biomass; previous studies have shown that bacteria use biofilm aggregation as a size-dependent defence mechanism against protist predators (Friman *et al.*, 2013; Friman and Laakso, 2011; Matz *et al.*, 2004). Bacterial biofilm growth was measured by adding 50 µl of 1% crystal violet solution to microplate wells and rinsed off with distilled water after 10 minutes. Crystal violet stained bacteria were dissolved in 96% ethanol and the amount of biofilm measured as OD at 600 nm (O'Toole and Kolter, 1998).

Measuring eco-evolutionary changes in prey communities

Changes in bacterial community diversity were estimated on the basis of colony morphology. SBW25 bacterium can rapidly diversify into different colony types by growing in the air-liquid interface (wrinkly spreader colony types), liquid media (smooth colony types) or by sinking to the bottom of the culture vessels (fuzzy spreader colony type) (Rainey and Travisano, 1998). All these colony types have fitness advantage when rare and can be maintained in the population via negative frequency-dependent selection (Rainey and Travisano, 1998). In addition to spatial heterogeneity,

protist predation can drive SBW25 diversification by favouring wrinkly spreader types (Meyer and Kassen, 2007), which differ genetically from ancestral smooth colony type (Spiers, 2014). We quantified bacterial diversification in the end of the experiment (last sampling point) by counting the number of different colony types from each treatment (plates containing at least 100 individual bacterial colonies). Prey population diversities were estimated with Shannon diversity index (Friman *et al.*, 2008). Prey population stability was determined by calculating the coefficient of variation for each replicate population by using whole time series: high coefficient denotes for higher variability (Friman *et al.*, 2008). Prey population productivity was measured as maximum densities in the absence of predators after 48 h growth at 22°C (200 μL of 0.5% LB medium).

Statistical analyses

A general linear mixed model (GLMM; Gaussian family) was used to analyse all data. In all models, the dependent variable was explained with experimental treatment, focal protist species, measurement environment, sampling time and their interactions. For repeated measures analyses, populations were set as subjects and time as a repeated factor. Replicates were nested under treatments and fitted as a random factor. Additional GLMMs were carried out when significant interactions were found. Log-transformed values were used for analysing protist densities due to unequal variances between the treatments. Arcsin-transformed values were used to analyse differences in colony type frequencies. Bonferroni-adjusted *P*-values were used for multiple pairwise comparisons.

RESULTS

(a) Predator effects on bacterial population dynamics

Only *T. pyriformis* and *T. vorax* reduced bacterial densities in single-predator treatments (treatment: $F_{4,\ 19.53} = 13.9$, $P < 0.001$, Fig. 1a-b), while *A. polyphaga* or *C. paramecium* had no effect on bacterial densities ($P = 0.365$ and $P = 0.183$, respectively, Fig 1c-d). The *T. pyriformis*-driven decrease in bacterial densities was attenuated only in the presence of *T. vorax* in both two- and four-

predator communities (treatment: $F_{5, 23.78} = 81.2$, $P < 0.001$; *A. polyphaga* or *C. paramecium* had no effect: $P = 0.559$ and $P = 0.456$, respectively, Fig. 1a). Similarly, the *T. vorax*-driven decrease in bacterial densities was attenuated in the presence of *T. pyriformis* but only in the two-predator communities (treatment: $F_{5, 21.99} = 23$, $P < 0.001$; *A. polyphaga* or *C. paramecium* had no effect: $P = 0.906$ and $P = 0.881$, respectively, Fig. 1b). Finally, the presence of *A. polyphaga* had no effect on *C. paramecium* and vice versa ($P = 0.158$ and $P = 0.600$, respectively, Fig. 1c-d).

Together these results show that only the two *Tetrahymena* species decreased bacterial densities, while this effect was constrained only by the presence of the other *Tetrahymena* species (summarised in Fig. 6).

(b) Predator effects on protist population dynamics

The dynamics of the predator communities are summarised in Fig. 2 and 6. *T. pyriformis* reached highest, *A. polypahaga* second highest, and *T. vorax* and *C. paramecium* reached lowest densities in single-predator treatments ($F_{3, 13.86} = 21.97$, $P < 0.001$, Fig. 2a-d). We observed several types of interaction among the protists, including negative, positive, and neutral interactions (focal protist density difference between single- and multi-protist treatments). Overall, *T. pyriformis* was little affected by the presence of the other species and grew well in all combinations except those in which *T. vorax* was present, where it was strongly depressed ($F_{4, 18} = 197.86$, $P < 0.001$). Similarly, *T. pyriformis* had a negative effect on *T. vorax* ($F_{4, 16.47} = 5.9$, $P = 0.004$). *C. paramecium* experienced a strong positive response to *T. pyriformis* (treatment × time: $F_{20, 14.59} = 6.25$, $P < 0.001$, Fig. 2c). Finally, *A. polyphaga* grew well on its own or in the presence of *C. paramecium*, but its growth was depressed by the two ciliates ($F_{4, 20.18} = 349.6$, $P < 0.007$).

(c) Bacterial defence evolution in single-predator and multi-predator communities

In single-predator communities, bacteria evolved defence to protist predation only in the presence of *T. pyriformis* ($F_{1, 8} = 15.9$, $P = 0.004$; none of the other protists increased bacterial defence in any single-predator treatments: all $P > 0.05$, Fig. 3). The *T. pyriformis* driven increase in bacterial

defence was affected by the presence of other protists ($F_{5, 24} = 5.65$, $P = 0.001$, Fig. 3a): concurrent selection by *T. vorax* repressed defence evolution in both two- and four-predator communities ($P < 0.001$ and $P = 0.007$, respectively), while bacterial defence against *T. pyriformis* also evolved less strongly in the presence of *C. paramecium* ($P = 0.039$; *A. polyphaga* had no effect: $P = 0.497$). Bacteria did not evolve defence against *T. vorax* or *C. paramecium* in any of the treatments (treatment for *T. vorax*: $F_{5, 24} = 2.7$, $P = 0.09$; treatment for *C. paramecium*: $F_{5, 24} = 1.96$, $P = 0.12$; Figs. 3b-c). However, bacteria evolved defence against *A. polyphaga* in the *A. polyphaga*+*T. pyriformis*, *A. polyphaga*+*T. vorax* and four-protist treatments ($F_{5, 24} = 11.56$, $P < 0.001$; $P < 0.03$ in all pairwise comparisons).

Together these results suggest that only *T. pyriformis* impose detectable selection for bacterial defence evolution in single-predator communities. In multi-protist communities, selection by *T. pyriformis* was attenuated in the presence of some other protists (*T. vorax* and *C. paramecium*), while in some cases bacteria evolved defence only in the presence of several protist species (e.g., *A. polyphaga*-ciliate treatments).

(d) Eco-evolutionary dynamics in single- and multi-predator communities

i) Predator-driven bacterial phenotypic diversification

Only *T. pyriformis* predation led to bacterial phenotypic diversification within single predator treatments (Shannon index; $F_{4, 20} = 61.36$, $P < 0.001$, Fig. 4a). Diversification was due to increase in the frequency of wrinkly spreader (WS; $F_{4, 16} = 35.96$, $P < 0.001$; 36% of all colonies), and petite colony types (PT; $P = 0.37$; 5% of all colonies; non-significant due to variation between replicates), resulting in decrease of ancestral, smooth colony type (SM; $F_{4, 20} = 97.26$, $P < 0.001$; 59% of all colonies vs. 100% of all colonies in bacterium-only treatment).

Bacterial diversification was further shaped by the presence of other enemies ($F_{4, 16} = 35.96$, $P < 0.001$, Fig. 4a). While *T. vorax* repressed diversification in the presence of *T. pyriformis* (Shannon index; $F_{5, 24} = 66.38$, $P < 0.001$; 100% of colonies SM type), both *C. paramecium* and *A. polyphaga* altered *T. pyriformis*-driven bacterial diversification by selecting for transparent colony

types (TT) that were not observed in the *T. pyriformis*-bacterium treatment (0% vs. 17% and 23% of all colonies, respectively). Similar to the *T. pyriformis*-only treatment, PT colony types (10% of all colonies) emerged also in the presence of *C. paramecium*, while no PT colony types were observed in the presence of *A. polyphaga*.

Together these results suggest that *T. pyriformis* was the main driver of bacterial phenotypic diversification, while this process was further promoted by both *C. paramecium* and *A. polyphaga* and completely repressed by *T. vorax*.

ii) Phenotypic diversification and evolution of different defence strategies

To assess whether bacterial phenotypic diversification was connected to evolution of different defensive strategies, we measured the defence of different bacterial colony types separately against all protist they had been exposed to during the selection experiment. WS colony types isolated from the *T. pyriformis* monocultures were clearly more defensive compare to SM colony types ($F_{2, 16.48} = 30.52$, $P < 0.001$, Fig. 4b). However, SM or PT colony types originating from the *T. pyriformis* monoculture treatment were equally poor at defending as SM colony types originating from bacterium-only treatment ($F_{1, 8.6} = 0.529$, $P > 0.05$ in both cases, Fig. 4b).

We next compared the defence of evolved bacteria originating from the *T. pyriformis*+*C. paramecium* treatment (Fig. 4c). We found that WS colony types evolved equal levels of defence in the *T. pyriformis* monoculture and the *T. pyriformis*+*C. paramecium* treatments ($F_{2, 56.54} = 1.41$, $P = 0.252$, Fig. 4c). WS colony types originating from *T. pyriformis*+*C. paramecium* treatment were only slightly better at defending against *C. paramecium* compared to SM colony types. This suggests that defence against *T. pyriformis* was traded-off with defence against *C. paramecium* (colony type × predator species: $F_{12, 42.07} = 6.87$, $P < 0.001$, Fig. 4c). The PT colony types were equally defensive against *C. paramecium* as the WS types (PT vs. SM: $P = 0.017$; PT vs. WS: $P = 0.952$, Fig. 4c). However, PT colony types were equally susceptible to *T. pyriformis* as SM colony types (PT vs. SM: $P = 0.912$, Fig. 4c), which suggests that PT types specialised to defend against *C.*

293 *paramecium*. The TT colony types that emerged in small frequency were not particularly good
294 defenders against any predator.
295 Finally, we assessed the defence of evolved bacteria originating from the *T. pyriformis*+*A.*
296 *polyphaga* treatment (Fig. 4d). We found that WS colony types evolved equally defensive in *T.*
297 *pyriformis* monoculture and *T. pyriformis*+*A. polyphaga* treatments ($F_{2, 56.54} = 1.41$, $P = 0.252$, Fig.
298 4d). WS colony types originating from the *T. pyriformis*+*A. polyphaga* treatment were also clearly
299 better at defending against *A. polyphaga* compared to ancestral SM colony types. This suggests that
300 defence against *T. pyriformis* correlated positively with defence against *A. polyphaga* (colony type
301 × predator species: $F_{12, 43.5} = 4.45$, $P < 0.001$, Fig. 4d). Moreover, TT colony types evolved higher
302 levels of defence against *A. polyphaga* (TT vs. SM: $P = 0.046$, Fig. 4d). However, this specialist
303 defence strategy correlated negatively with defence against *T. pyriformis*: TT colony types were as
304 susceptible to *T. pyriformis* as ancestral SM colony types (TT vs. SM: $P = 0.517$).
305 These results suggest that *T. pyriformis* selected for generalist defenders in two-predator
306 communities (WS colony types) that were highly defended against both enemies they had been
307 exposed to during the selection experiment. Furthermore, *C. paramecium* and *A. polyphaga* selected
308 for specialist defenders in two-predator communities (PT and TT colony types, respectively) that
309 were poor at defending against *T. pyriformis* but good at defending against *C. paramecium* and *A.*
310 *polyphaga*, respectively.
311
312 ***iii) Changes in stability and productivity of prey populations***
313 Prey selection lines that evolved defence against protists (*T. pyriformis* monoculture, *T.*
314 *pyriformis*+*A. polyphaga* and *T. pyriformis*+*C. paramecium*) became temporally more variable
315 compared to the control selection line (bacterium alone) or selection lines that did not evolve
316 defence against any protists ($F_{1, 50} = 14.6$, $P < 0.001$; $P < 0.001$ in all pairwise comparisons) in both
317 single and two-predator communities ($F_{1, 50} = 0.004$, $P = 0.95$; Fig. 5a). Non-evolved and control
318 selection lines were equally variable ($P = 0.2$). Similarly, prey selection lines that evolved defence
319 against protists became less productive compared to control selection line or selection lines that did

not evolve defence against any protists ($F_{1, 50}$ = 7.7, P < 0.001; P < 0.001 in all pairwise comparisons) in both single- and two-predator communities ($F_{1, 50}$ = 0, P = 0.98; Fig. 5b). Non-evolved and control selection lines were equally productive (P = 0.8). At the colony type level, reduced productivity was due to poorer growth of WS, PT and TT colony types relative to ancestral-like SM colony types ($F_{3, 28}$ = 4.41, P = 0.012; P < 0.05 in all pairwise comparisons; Fig. 5c). Of specialist defenders, TT colony type suffered highest reduction in growth (WS vs TT: P = 0.018), while PT colony types suffered intermediate reduction in growth (WS vs PT: P = 0.216 and TT vs PT: P = 0.27; Fig. 5c).

DISCUSSION

Here we studied experimentally the role of predator species identity and community complexity for the prey population dynamics, prey defence evolution and potential ecological feedbacks. We found that *T. pyriformis* was a key driver of defence evolution in both single- and two-protist communities. While other protists did not select for prey defence in single-protist treatment, concurrent selection by *T. pyriformis* and *C. paramecium* and *T. pyriformis* and *A. polyphaga* led to evolution of specialised defence strategies. Prey defence evolution was repressed in the presence of the intraguild predator, *T. vorax*, which was able to efficiently feed on *T. pyriformis* cells in both two-predator and four-predator communities. At the ecological level, adapted prey populations became phenotypically more diverse, less stable and less productive compared to non-adapted prey populations. Together these results suggest that increasing predator community richness can increase prey diversity via selection for specialist defence strategies. However, introduction of intraguild top-predator tipped the balance from evolutionary to purely ecological community dynamics. Predator-prey interactions are thus more likely to evolve in communities with weak predator-predator interactions.

T. pyriformis was the only predator species that significantly reduced prey populations, and was the only predator consistently associated with the evolution of prey defence and diversification. These results are broadly consistent with previous studies (Friman and Buckling, 2013; Friman *et*

al., 2014; Meyer and Kassen, 2007). *C. paramecium* and *A. polyphaga* were more weakly linked with prey bacteria and did not significantly decrease bacterial densities in single-protist cultures, which could also explain relatively weak selection for prey defence. Bacteria did not evolve detectable defence against *T. vorax* either in single-protist cultures, despite the clear reduction in bacterial densities. One explanation for this could be that large *T. vorax* (maximum cell length of ~200 µm) were able to effectively consume bacterial biofilm aggregates due to their larger orifice, while the relatively smaller *T. pyriformis* (~60 µm in cell length) were not.

Even though *C. paramecium* and *A. polyphaga* did not select for detectable changes in prey defence in single-protist cultures, they affected the diversification of bacterial defensive strategies in *T. pyriformis* co-cultures. First, the frequency of wrinkly colony types (WS) increased *T. pyriformis*, *T. pyriformis+C. paramecium* and *T. pyriformis+A. polyphaga* treatments. This is in line with previous studies where predation by *T. pyriformis* and *T. thermophila*, a closely related species (Brunk *et al.*, 2003), has been shown to drive bacterial diversification in defensive phenotypes (Friman and Buckling, 2014; Meyer and Kassen, 2007; Mikonranta *et al.*, 2012). WS colony types were equally defensive against *T. pyriformis* regardless if they had evolved in the presence of *C. paramecium* or *A. polyphaga*. Similarly, WS colony types that emerged in two-protist treatments were able to defend against *C. paramecium* and *A. polyphaga* compared to non-defending SM colony types. This suggests that WS colony types exerted generalist defence strategy. Moreover, bacteria diversified into petite (PT) and transparent (TT) colony types in *T. pyriformis+C. paramecium* and *T. pyriformis+A. polyphaga* treatments. These colony types were specialised to defend against *C. paramecium* and *A. polyphaga*, but were at the same time susceptible to predation by *T. pyriformis*. As a result, concurrent selection by two different protists led to coexistence of generalist and specialist defenders (Berenbaum and Zangerl, 2006; Friman and Buckling, 2013; Parchman and Benkman, 2008), resulting in increased intra-bacterial diversity. Even though these specialist defenders (PT and TT) had a fitness advantage over the non-defending SM colony types at least in the presence of one predator, they always had lower or equally high fitness with a generalist defender (WS). Why were not these specialists driven into extinction? One

possibility is that, by testing each colony type in isolation, we have not accounted for interactions with the other colony types. Alternatively, slow-growing PT and TT colony types could have been organized in the bottom of mixed biofilms resulting in enhanced protection against protist predation (Kim *et al.*, 2014), or could have hitchhiked along with SM and WS colony types in the mixed biofilms (Friman *et al.*, 2013; Popat *et al.*, 2012). While further experiments are needed to test these hypotheses, our results suggest that concurrent selection by two protists potentially changes the topology of bacterial fitness landscape in ways that allow bacterial adaptation against multiple enemies (Flynn *et al.*, 2013).

We also found that protists had negative, positive and neutral effects on each other in multi-protist cultures. While both *T. pyriformis* and *T. vorax* ciliates reduced bacterial densities efficiently in the absence of other predators, their independent effects were attenuated in the presence of each other. This can be explained by indirect and direct interference. First, *T. pyriformis* likely reduced the *T. vorax* effect on bacterial prey by indirectly competing for the same bacterial resource. Second, macrostome morphs of *T. vorax* can directly consume *T. pyriformis* (Banerji and Morin, 2009), which could have reduced *T. pyriformis* densities leading to weakened selection for bacterial defence. Defence evolution against *T. pyriformis* was also weakened in the presence of *C. paramecium*. As *C. paramecium* did not affect *T. pyriformis* densities in cocultures, this result is more likely explained by the evolution of specialist defenders that were weakly defended against *C. paramecium* (PT and TT colony types). Unexpectedly, *T. pyriformis* enhanced *C. paramecium* growth. Even though the mechanism for this is unknown, one explanation could be that *C. paramecium* was able to cross feed on *T. pyriformis* waste metabolites – a common process often observed between different bacteria (Lawrence *et al.*, 2012). We also found that concurrent selection by *A. polyphaga* and *T. pyriformis*, or *A. polyphaga* and *T. vorax*, led to increased bacterial defence against *A. polyphaga*. Together these results suggest that protist predators can exert conflicting or diffuse selection (Janzen, 1980; Strauss and Irwin, 2004) leading to specialist or generalist defensive strategies in multi-predator communities.

In addition to increased bacterial phenotypic diversity, prey defence evolution changed other ecological aspects of predator-prey communities. First, evolved prey populations were more variable in time (higher coefficient of variation) compared to non-evolved or control populations. Prey defence evolution can destabilise predator-prey dynamics for example by changing the amplitude and phase of predator-prey cycles (Abrams, 2000; Becks *et al.*, 2010; Yoshida *et al.*, 2003). Moreover, competitive interactions between different prey phenotypes could increase population instability via frequency-dependent selection (Meyer and Kassen, 2007; Yoshida *et al.*, 2003). Unfortunately, we cannot separate these hypotheses with our data, as we quantified evolutionary changes only in the end of the experiment. We also found that evolved prey populations were equally variable in single-predator and two-predator communities even though some two-predator communities had higher phenotypic prey richness (*T. pyriformis*- *C. paramecium*). This suggests that relatively more abundant SM and WS colony types were associated with the largest effect on destabilization of evolved prey populations. We also found that evolved prey populations became less productive compared to non-evolved or control populations. At the colony-type level, reduced growth was linked with specialist and generalist defender prey phenotypes. This suggests that evolving defence was traded-off with prey competitive ability, a commonly found trade-off in microbial predator-prey systems (Friman *et al.*, 2015; Friman and Laakso, 2011; Friman and Buckling, 2013; Meyer and Kassen, 2007; Yoshida *et al.*, 2003). Such trade-off could also have affected prey population instability (Abrams, 2000; Ellner and Becks, 2011; Yoshida *et al.*, 2003). Together these results suggest that multiple predators can have emergent evolutionary effects on prey that cannot be predicted on the basis of pairwise interactions.

To conclude, our results show that predator community composition is important in defining the relative importance of ecological and evolutionary dynamics of microbial communities. In general, increasing protist community richness increased prey diversity by allowing the evolution of specialist defence strategies. However, ecological dynamics dominated in the presence of top-predator due to reduction in the densities of *T. pyriformis* – a key driver of bacterial adaptation. Intraguild predation could thus indirectly constrain evolution of predator-prey interactions.

ACKNOWLEDGEMENTS

This study was funded by Imperial College Junior Research Fellow scheme (VF), Grant-in-Aid funding from The Natural History Museum (DB), NHM-UCL PhD studentship (AD), and the Royal Society and the Natural Environment Research Council (TB).

References

Abrams PA (2000). The evolution of predator-prey interactions: theory and evidence. *Annu Rev Ecol Syst***:** 79-105.

Banerji A, Morin PJ (2009). Phenotypic plasticity, intraguild predation and anti-cannibal defences in an enigmatic polymorphic ciliate. *Funct Ecol* **23:** 427-434.

Becks L, Ellner SP, Jones LE, Hairston NG, Jr. (2010). Reduction of adaptive genetic diversity radically alters eco-evolutionary community dynamics. *Ecol Lett* **13:** 989-997.

Berenbaum MR, Zangerl AR (2006). Parsnip webworms and host plants at home and abroad: Trophic complexity in a geographic mosaic. *Ecology* **87:** 3070-3081.

Billick I, Case TJ (1994). Higher-Order Interactions in Ecological Communities - What Are They and How Can They Be Detected. *Ecology* **75:** 1529-1543.

Brunk CF, Lee LC, Tran AB, Li JL (2003). Complete sequence of the mitochondrial genome of Tetrahymena thermophila and comparative methods for identifying highly divergent genes. *Nucleic Acids Res* **31:** 1673-1682.

Craig TP, Itami JK, Horner JD (2007). Geographic variation in the evolution and coevolution of a tritrophic interaction. *Evolution* **61:** 1137-1152.

Davies N, Brooke ML (1989). An experimental study of co-evolution between the cuckoo, Cuculus canorus, and its hosts. I. Host egg discrimination. *J Anim Ecol***:** 207-224.

Edeline E, Ben Ari T, Vollestad LA, Winfield IJ, Fletcher JM, Ben James J *et al* (2008). Antagonistic selection from predators and pathogens alters food-web structure. *PNAS* **105:** 19792-19796.

Elliott AM (1959). Biology of Tetrahymena. *Annu Rev Microbiol* **13:** 79-96.

Ellner SP, Becks L (2011). Rapid prey evolution and the dynamics of two-predator food webs. *Theor Ecol-Neth* **4:** 133-152.

Flynn KM, Cooper TF, Moore FB, Cooper VS (2013). The environment affects epistatic interactions to alter the topology of an empirical fitness landscape. *PLoS Genet* **9:** e1003426.

Fox LR (1988). Diffuse Coevolution within Complex Communities. *Ecology* **69:** 906-907.

Friman V-P, Diggle SP, Buckling A (2013). Protist predation can favour cooperation within bacterial species. *Biol Lett* **9**.

Friman V-P, Buckling A (2014). Phages can constrain protist predation-driven attenuation of Pseudomonas aeruginosa virulence in multienemy communities. *ISME J*.

Friman V-P, Guzman LM, Reuman DC, Bell T (2015). Bacterial adaptation to sublethal antibiotic gradients can change the ecological properties of multitrophic microbial communities. *Proceedings of the Royal Society of London B: Biological Sciences* **282:** 20142920.

Friman VP, Hiltunen T, Laakso J, Kaitala V (2008). Availability of prey resources drives evolution of predator-prey interaction. *Proceedings Biological sciences / The Royal Society* **275:** 1625-1633.

Friman VP, Laakso J (2011). Pulsed-resource dynamics constrain the evolution of predator-prey interactions. *Am Nat* **177:** 334-345.

Friman VP, Buckling A (2013). Effects of predation on real-time host-parasite coevolutionary dynamics. *Ecol Lett* **16:** 39-46.

Friman VP, Jousset A, Buckling A (2014). Rapid prey evolution can alter the structure of predator–prey communities. *J Evolution Biol* **27:** 374-380.

Futuyma DJ, Moreno G (1988). The Evolution of Ecological Specialization. *Annu Rev Ecol Syst* **19:** 207-233.

Gomez JM, Perfectti F, Bosch J, Camacho JPM (2009). A geographic selection mosaic in a generalized plant-pollinator-herbivore system. *Ecol Monogr* **79:** 245-263.

Gronlien HK, Berg T, Lovlie AM (2002). In the polymorphic ciliate Tetrahymena vorax, the non-selective phagocytosis seen in microstomes changes to a highly selective process in macrostomes. *J Exp Biol* **205:** 2089-2097.

Hiltunen T, Jones LE, Ellner SP, Hairston NG (2013). Temporal dynamics of a simple community with intraguild predation: an experimental test. *Ecology* **94:** 773-779.

Hiltunen T, Becks L (2014). Consumer co-evolution as an important component of the eco-evolutionary feedback. *Nature communications* **5**.

509 Iwao K, Rausher MD (1997). Evolution of plant resistance to multiple herbivores: quantifying
510 diffuse coevolution. *Am Nat***:** 316-335.

511
512 Janzen DH (1980). When Is It Coevolution. *Evolution* **34:** 611-612.

513
514 Kim W, Racimo F, Schluter J, Levy SB, Foster KR (2014). Importance of positioning for microbial
515 evolution. *PNAS* **111:** E1639-1647.

516
517 Lawrence D, Fiegna F, Behrends V, Bundy JG, Phillimore AB, Bell T *et al* (2012). Species
518 Interactions Alter Evolutionary Responses to a Novel Environment. *PLoS Biol* **10**.

519
520 Matz C, Bergfeld T, Rice SA, Kjelleberg S (2004). Microcolonies, quorum sensing and cytotoxicity
521 determine the survival of Pseudomonas aeruginosa biofilms exposed to protozoan grazing. *Environ*
522 *Microbiol* **6:** 218-226.

523
524 Meyer JR, Kassen R (2007). The effects of competition and predation on diversification in a model
525 adaptive radiation. *Nature* **446:** 432-435.

526
527 Mikonranta L, Friman VP, Laakso J (2012). Life history trade-offs and relaxed selection can
528 decrease bacterial virulence in environmental reservoirs. *PloS ONE* **7:** e43801.

529
530 Nuismer SL, Thompson JN (2006). Coevolutionary alternation in antagonistic interactions.
531 *Evolution* **60:** 2207-2217.

532
533 O'Toole GA, Kolter R (1998). Initiation of biofilm formation in Pseudomonas fluorescens WCS365
534 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* **28:** 449-
535 461.

536
537 Parchman TL, Benkman CW (2008). The geographic selection mosaic for ponderosa pine and
538 crossbills: A tale of two squirrels. *Evolution* **62:** 348-360.

539
540 Patterson DJ (1996). *Free-living freshwater protozoa : a colour guide*: New York.

541
542 Popat R, Crusz SA, Messina M, Williams P, West SA, Diggle SP (2012). Quorum-sensing and
543 cheating in bacterial biofilms. *Proceedings Biological sciences / The Royal Society* **279:** 4765-4771.

544
545 Rainey PB, Bailey MJ (1996). Physical and genetic map of the Pseudomonas fluorescens SBW25
546 chromosome. *Mol Microbiol* **19:** 521-533.

547
548 Rainey PB, Travisano M (1998). Adaptive radiation in a heterogeneous environment. *Nature* **394:**
549 69-72.

550

Sih A, Englund G, Wooster D (1998). Emergent impacts of multiple predators on prey. *Trends Ecol Evol* **13:** 350-355.

Spiers AJ (2014). A Mechanistic Explanation Linking Adaptive Mutation, Niche Change, and Fitness Advantage for the Wrinkly Spreader. *International Journal of Evolutionary Biology* **2014:** 10.

Stinchcombe JR, Rausher MD (2001). Diffuse selection on resistance to deer herbivory in the ivyleaf morning glory, Ipomoea hederacea. *Am Nat* **158:** 376-388.

Strauss SY, Irwin RE (2004). Ecological and evolutionary consequences of multispecies plant-animal interactions. *Annu Rev Ecol Evol S* **35:** 435-466.

Strauss SY, Sahli H, Conner JK (2005). Toward a more trait-centered approach to diffuse (co)evolution. *New Phytol* **165:** 81-89.

Thompson JN, Cunningham BM (2002). Geographic structure and dynamics of coevolutionary selection. *Nature* **417:** 735-738.

Thompson JN (2005). *The geographic mosaic of coevolution*. University of Chicago Press: Chicago.

Vandermeer JH (1969). The Competitive Structure of Communities: An Experimental Approach with Protozoa. *Ecology* **50:** 362-371.

Yoshida T, Jones LE, Ellner SP, Fussmann GF, Hairston NG (2003). Rapid evolution drives ecological dynamics in a predator-prey system. *Nature* **424:** 303-306.

TITLES AND LEGENDS TO FIGURES

Figure 1. Bacterial population densities in different experimental communities (panels a-d). Abbreviations in the panels denote for SBW25 *P. fluorescens* bacterium (B), *T. pyriformis* (TP), *T. vorax* (TV), *C. paramecium* (CP) and *A. polyphaga* (AP) protists. All data points show mean of five replicate populations and ±1 SEM.

Figure 2. Protist population densities in different experimental communities (panels a-d). Abbreviations in the panels denote for SBW25 *P. fluorescens* bacterium (B), *T. pyriformis* Ciliate

588 (TP), *T. vorax* ciliate (TV), *C. paramecium* Flagellate (CP) and *A. polyphaga* amoebae (AP)
589 protists. All data points show mean of five replicate populations and ±1 SEM.
590
591 **Figure 3. Bacterial defence measured against *T. pyriformis* (a), *T. vorax* (b), *C. paramecium* (c)**
592 **and *A. polyphaga* (d) protists for bacteria originating from different experimental treatments**
593 **after the selection experiment**. Bacterial defence is calculated as the relative growth of protist-
594 evolved vs. alone-evolved bacterial populations. Abbreviations in the panels denote for *T.*
595 *pyriformis* (TP), *T. vorax* (TV), *C. paramecium* (CP) and *A. polyphaga* (AP) protists and white bars
596 denote single-predator, light grey bars two-predator, and dark grey bars four-predator communities.
597 All data points show mean of five replicate populations and ±1 SEM.
598
599 **Figure 4. Protist-driven bacterial phenotypic diversification (a) and the evolution of different**
600 **defence strategies in phenotypically diverse experimental communities (b-d).** Abbreviations in
601 the panels denote for SBW25 bacterium (B), *T. pyriformis* (TP), *T. vorax* (TV), *C. paramecium*
602 (CP) and *A. polyphaga* (AP) protists, smooth colony type (SM), wrinkly spreader colony type
603 (WS), transparent colony type (TT) and petite colony type (PT). In panel (a), left and right Y-axes
604 show colony type frequencies and Shannon diversity index, respectively. Panels (b-d) show WS, PT
605 and TT colony types' defence relative to SM colony types within *T. pyriformis*-only (b), *T.*
606 *pyriformis*+*C. paramecium* (c) and *T. pyriformis*+*A. polyphaga* (d) experimental treatments.
607 Colony types' defence was measured in the presence of *T. pyriformis* (TP), *C. paramecium* (CP) and
608 *A. polyphaga* (AP) protists. All data points show mean of five replicate populations and ±1 SEM.
609
610 **Figure 5. Comparison of prey population stability (a) and productivity (b-c) after selection**
611 **experiment.** In panels (a) and (b), grey bars show means for evolved treatments (*T. pyriformis*-only,
612 *T. pyriformis*+*A. polyphaga* and *T. pyriformis*+*C. paramecium*) and white bars show means for
613 non-evolved treatments (all other protist communities). X-axis in panels (a) and (b) denotes for the
614 number of protists prey selection lines evolved with during the selection experiment; white bar with

615 0 protists denote for control selection line (bacterium-only). Panel (c) shows productivity at the
616 colony type level within phenotypically most diverse experimental communities. Abbreviations in
617 all panels denote for SBW25 bacterium (B), *T. pyriformis* (TP), *C. paramecium* (CP), *A. polyphaga*
618 (AP) protists, smooth bacterial colony type (SM), wrinkly spreader bacterial colony type (WS),
619 transparent bacterial colony type (TT) and petite bacterial colony type (PT). In all panels, error
620 estimate is ±1 SEM.
621
622 **Figure 6. Schematic description of the eco-evolutionary dynamics observed during the**
623 **selection experiment in pairwise predator-prey communities (a), two predator-one prey**
624 **communities (b) and four predator-one prey communities (c).** In all panels, blue and red solid
625 lines denote for negative and positive effects on species population dynamics, respectively, black
626 dashed lines depict for bacterial defence evolution against given protist predators and pie charts
627 depict relative protist abundances. Pairwise predator-prey and two predator-one prey communities
628 were characterised by both ecological and evolutionary dynamics, while four predator-one prey
629 communities were dominated by ecological dynamics. Abbreviations in the panels denote for
630 SBW25 bacterium (B), *T. pyriformis* (TP), *T. vorax* (TV), *C. paramecium* (CP) and *A. polyphaga*
631 (AP) protists.