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# Dual-stressor selection alters eco-evolutionary dynamics in experimental communities

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**Recognizing when and how rapid evolution drives ecological change is fundamental for our understanding of almost all ecological and evolutionary processes such as community assembly, genetic diversification and the stability of communities and ecosystems. Generally, rapid evolutionary change is driven through selection on genetic variation and is affected by evolutionary constraints, such as tradeoffs and pleiotropic effects, all contributing to the overall rate of evolutionary change. Each of these processes can be influenced by the presence of multiple environmental stressors reducing a population's reproductive output. Potential consequences of multistressor selection for the occurrence and strength of the link from rapid evolution to ecological change are unclear. However, understanding these is necessary for predicting when rapid evolution might drive ecological change. Here we investigate how the presence of two stressors affects this link using experimental evolution with the bacterium *Pseudomonas fluorescens* and its predator *Tetrahymena thermophila*. We show that the combination of predation and sublethal antibiotic concentrations delays the evolution of anti-predator defence and antibiotic resistance compared with the presence of only one of the two stressors. Rapid defence evolution drives stabilization of the predator–prey dynamics but this link between evolution and ecology is weaker in the two-stressor environment, where defence evolution is slower, leading to less stable population dynamics. Tracking the molecular evolution of whole populations over time shows further that mutations in different genes are favoured under multistressor selection. Overall, we show that selection by multiple stressors can significantly alter eco-evolutionary dynamics and their predictability.**

Microbes often adapt surprisingly fast to changes in their environment. For instance, the rapid adaptation of resistance against pesticides or antibiotics[1,2] as well as the coevolution of interacting microbes[3–5] suggest an abundant supply of adaptive genetic variation. It is now well established that the dynamics of rapid evolutionary change can determine the ecological dynamics of populations and communities, which can again alter further evolutionary change and so on[6–8]. Because microbial communities determine the functioning of nearly all ecosystems[9], understanding their eco-evolutionary dynamics is of fundamental importance, for example, for predicting harmful bacterial blooms[10], the community composition of the holobiont[11] or the potential of a microbial community to serve as a reservoir for antibiotic resistance alleles[2].

Recent work has uncovered important consequences of eco-evolutionary dynamics, for example, for the coexistence of interacting species[12], temporal changes in their population sizes[6] and the maintenance of diversity[3,13]. Eco-evolutionary dynamics and their consequences are typically studied in the presence of one environmental stressor that leads to a reduction in fitness (for example, one consumer or the exposure to antibiotics). However, the underlying mechanisms linking evolutionary and ecological change are virtually unknown in communities with more than one stressor (for example, consumer and antibiotics). Previous work has examined multistressor selection[14,15], but this work has been limited to investigations of the evolutionary or ecological dynamics rather than the links between ecology and rapid evolution. One important question with multiple stressors is whether or not the same links between evolution and ecology matter as with they do one stressor. Here we develop predictions for the link between the evolutionary and ecological dynamics in single- and multiple-stressor environments and test these in an experimental evolution study. We focus on two common-place stressors in microbial communities—ciliate predation and sublethal antibiotic concentrations (sub-minimum inhibitory concentrations (sub-MICs))—and disentangle for the first time key processes driving the link between ecological and evolutionary dynamics in bacteria–ciliate communities.

Sub-MIC levels are commonly found, for example, in sewage waters, lakes, rivers and soil[16], and they have been shown to select for antibiotic resistance either by an increase in the frequency of resistant bacteria or by selection for de novo resistance[1]. Besides evolutionary consequences, sub-MICs of antibiotics can also affect ecological dynamics, for example, by lowering bacterial population sizes when sub-MICs of antibiotics do not alter growth rates but increase density-independent mortality rates[2]. From these observations, we can further predict a direct link between evolutionary and ecological dynamics when resistance evolution leads to higher growth rates or compensation for increased death rates in the presence of sub-MICs such that bacteria reach similar densities to those without sub-MICs.

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Consumption by protists or phages exerts strong selection on the bacterial prey population apart from the ecological effect of driving bacterial population. Bacteria are known to rapidly evolve anti-predatory adaptations against consumers, for example by evolving to grow in colonies or as biofilm, thereby decreasing attack rates or increasing handling time[16,17]. General ecological theory for predator–prey interactions predicts that decreasing attack rates and/or large increases in handling time can result in the stabilization of the temporal dynamics of the prey and its consumer[18], which can be seen in oscillations with reduced amplitudes or a shift to steady-state dynamics (Supplementary Fig. 1; note that the conditions for stability depend on the details of the model applied, for example the functional response of the predator)[19]. Thus the evolution of defence traits can directly affect the ecological dynamics within predator–prey systems, which has been confirmed in models[20] and experiments[21–23].

Sub-MIC levels of antibiotics can, however, alter the evolution of anti-consumer defence traits in bacterial populations[4,24], and the presence of the two stressors also has the potential to alter the stability of the microbial community (ecology). Multistressor selection can prevent or delay the evolution of resistance and/or anti-predatory defences through lowering the selection strength on individual loci[14], clonal interference where adaptive mutations compete for fixation in large asexual populations[25], tradeoffs between traits[26,27], pleiotroy[28] or linkage disequilibrium[29]. Furthermore, the evolution of one trait can alter the strength of species interactions and thus selection, which can lead to slower evolution, or favour different mutations due to differences in associated costs or because the role of the order of mutations changes in different environments. Bacterial population sizes are predicted to be lower in the presence of the two stressors as the combined effect lowers fitness even more, which can affect evolutionary change by reducing mutation supply and increasing the relative importance of drift to selection[30,31]. Alternatively, pleiotropic effects of mutations might accelerate the evolution of one trait when adaption to one stressor provides adaption to the second one at the same time. The effects of clonal interference could be alleviated in small population sizes, as clonal interference occurs less often when mutation supply is low[32,33]. The pace of evolution is also predicted to be faster when the predator removes selectively maladapted individuals or through the evolutionary hydra effect[34]. Finally, we predict that differences in the rate of evolution impact the population dynamics of the bacterial prey and the predator, with slower evolution leading to less stable and faster evolution to more stable predator–prey dynamics under the assumption of stabilizing selection[35].

To test for the role of multistressor selection for eco-evolutionary dynamics, in an experimental evolution study we exposed initially isogenic populations of the bacterium *Pseudomonas fluorescens* SBW25 to 0×MIC and 0.1×MIC of the antibiotic streptomycin (mode of action: inhibition of protein synthesis in prokaryotes) in the presence and absence of the ciliate *Tetrahymena thermophila* in a full-factorial experiment for ~220 bacterial and ciliate generations (66 days; see Methods). We followed the population dynamics and the phenotypic changes of three replicated populations in each treatment. To gain mechanistic insights into how sub-MICs and predation altered the evolution of defence and resistance, we analysed whole-genome sequence data from the replicate bacterial populations over time. This allowed us to compare when de novo mutations (single nucleotide polymorphisms (SNPs); insertions or deletions (INDELs); copy number variations (CNVs)) arise and their dynamics over time across the different treatments.

## Results and discussion

We observed different ecological and evolutionary dynamics over time depending on the presence/absence of the ciliates, as well as between treatments with the presence of streptomycin (Fig. 1). Streptomycin did not have a direct effect on the maximum growth rates of ciliates and bacteria (Supplementary Fig. 2). However, bacterial densities were significantly lower with streptomycin (generalized estimating equations model (GEE) bacteria alone: sub-MIC: $W=19.11$, d.f.$=1$, $P=1.236\times10^{-5}$; for all non-significant results, see Supplementary Information and Supplementary Table 1) as well as in the presence of ciliates (GEE: interaction sub-MIC×day: $W=4.96$, d.f.$=1$, $P=0.026$; day: $W=29.54$, d.f.$=1$, $P=5.47\times10^{-8}$; sub-MIC: $W=61.68$, d.f.$=1$, $P=3.997\times10^{-15}$). Overall, population dynamics were less stable in the presence of the ciliates and even less in the presence of ciliates with streptomycin (de-trended standard variation of the predator population = coefficient of variation: generalized linear model (GLM): $F=16.963$, d.f.$=2$, $P=0.0146$; Figs. 1 and 2a). Bacteria–ciliate populations showed considerable fluctuations at the beginning of the experiment in the presence and absence of streptomycin, but stabilized in the latter case around day 25. In the predator-free treatments, bacterial population sizes showed only small fluctuations around the carrying capacity (Fig. 1a,b). Thus, the sub-MIC and the presence of the predator led to lower bacterial population sizes and the predator to less stable dynamics, which was stronger with sub-MIC streptomycin.

To follow the evolutionary response of predation by the ciliate, we measured the growth rates $r$ of the ancestral predator when growing on ancestral and evolved bacteria isolated from different time points of the experiment. From this we calculated the defence level $D=\left(1-\frac{r_{\text{evolved}}}{r_{\text{ancestor}}}\right)$, with the value 0 meaning that the evolved bacteria have the same level of defence as the ancestor and values close to 1 meaning a very high level of defence compared to the ancestor[17]. Bacteria evolved anti-predatory defence by forming biofilm and/or colonies (Supplementary Fig. 3), with significantly higher levels of defence with 0×MIC levels over time (GEE: day: $W=13.03$, d.f.$=1$, $P=0.00031$; sub-MIC: $W=15.38$, d.f.$=1$, $P=8.81\times10^{-5}$; Fig. 1b,d; Supplementary Table 2). Lower predator growth rates were attributed to significantly lower ingestion rates for the defended prey compared to the undefended ancestral prey (analysis of variance starting concentrations versus ingestion: interaction concentration×defence: $F_{1,60}=11.44$, $P=0.001$; concentration: $F_{1,60}=76.67$, $P=7\times10^{-12}$; defence: $F_{1,60}=20.86$, $P=2.51\times10^{-5}$; Supplementary Fig. 4), which could be the result of lower attack rates or increased handling times (Supplementary Fig. 1).

We found the evolution of streptomycin resistance in populations in the predator-free environments with 0.1×MIC (Fig. 1c), which we confirmed by testing individual isolates from the end of the experiment (GLM for the comparison ancestor versus isolates from the end of the experiment: $F=37.6$, d.f.$=8$, $P=4.6\times10^{-5}$; Supplementary Fig. 5). Importantly, however, streptomycin resistance was not observed in the 0.1×MIC populations with predators (GLM for the comparison ancestor versus isolates from the end of the experiment with family: $F=2.32$, d.f.$=8$, $P=0.15$; Fig. 1d and Supplementary Fig. 5). To test whether resistance evolution was delayed or not occurring, we followed the frequency of resistance evolution in 48 additional populations with a factorial design including the presence and absence of ciliates in 0.1×MIC streptomycin (Methods). We found an increase in the frequency of resistant populations within 16 days in all treatments but the overall level of resistance was lower in the presence of the ciliates in 0.1×MIC (GEE: interaction day×treatment: $W=35.46$, d.f.$=3$, $P=9.738\times10^{-8}$; treatment: $W=440.5$, d.f.$=1$, $P<2.2\times10^{-16}$; day: $W=14.15$, d.f.$=1$, $P=0.00014$; Supplementary Fig. 6). Thus, resistance and defence evolution were delayed in the presence of the two stressors.

Next, we investigated the links between the ecological and evolutionary dynamics over time and across different environments. Bacterial population sizes were significantly lower in the presence of 0.1×MIC streptomycin even after they evolved resistance (Fig. 1a,c)

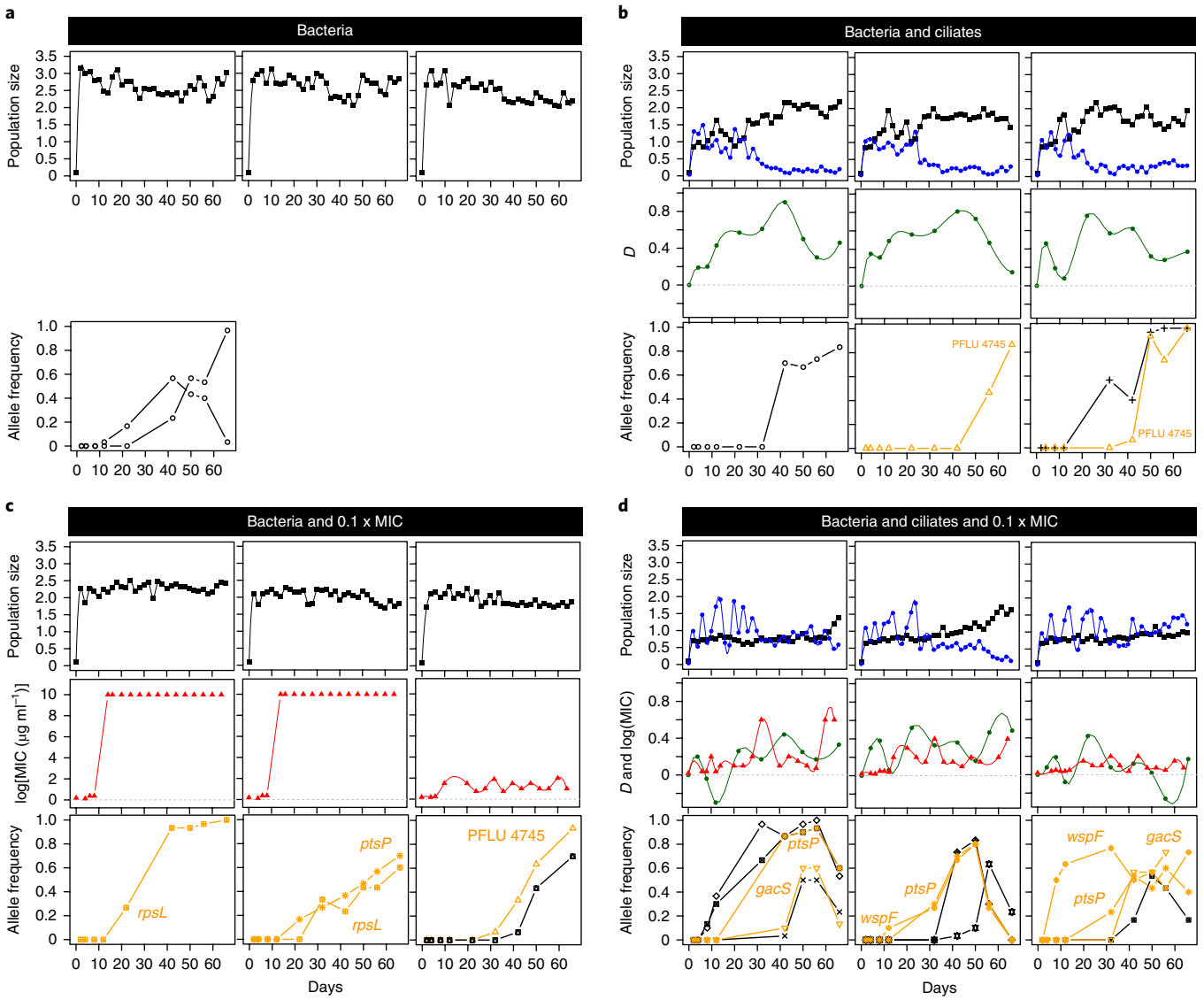


**Fig. 1 | Eco-evolutionary dynamics of *P. fluorescens* populations exposed to sub-MIC levels of streptomycin and ciliates. a–d**, Prey (**a**,**c**, top), predator–prey (**b**,**d**, top), prey defence *D* (middle), resistance (middle) and derived allele frequency (bottom) dynamics from *P. fluorescens* populations exposed to sub-MIC levels of streptomycin. Shown are three replicates 1–3 (left to right) per treatment: only bacteria (**a**); bacteria and ciliates (**b**); bacteria and 0.1 × MIC streptomycin (**c**); and bacteria and ciliate and 0.1 × MIC streptomycin (**d**). Bacteria are at $10^8$ cells per ml (black squares), ciliates are at $10^4$ cells per ml (blue circles), defence level *D* (green circles), resistance (log[MIC (μg ml$^{-1}$)], red triangles). Derived alleles are shown in black and different symbols when only found in one population (in orange) and with the same symbol when found in more than one population. We only show trait data (defence, log(MIC)) for treatments where they were collected, and when derived alleles passed filtering steps and reached at least 50% frequency in non-mutator populations (Methods). Note that the log(MIC) values are higher in **c** and that 10 is the maximum we could measure.

suggesting that the evolution of resistance had no effect on the ecological dynamics of the system. We found a significant negative correlation between the stability of the bacteria–ciliate communities and the defence level (GLM: mean defence level: $F=21.96$, d.f.$=1$, $P=0.0094$; Fig. 2b) suggesting that the evolution of defence altered the predator–prey dynamics as predicted by ecological theory (Supplementary Fig. 1). To further test whether the degree of defence alters the stability of the predator–prey system, we repeated the experiment but starting with clonal bacterial lineages differing in their defence level in the presence and absence of 0.1 × MIC streptomycin (Methods). Again, we found a significant negative correlation between stability and defence level but it was independent of the presence of sub-MIC levels of streptomycin (GLM: level of defence of initial clone: $F=14.06$, d.f.$=1$, $P=0.00057$; Fig. 2c). Thus the evolution of defence altered the ecological dynamics of predator and prey and, importantly, the presence of sub-MIC streptomycin did not directly affect the predator–prey dynamics but rather it had an indirect effect by slowing down defence evolution. Thus the relative role of defence evolution for the predator growth was lower in the presence of sub-MIC streptomycin compared to the ecological change, that is, the number of available prey (ratio of evolutionary change to ecological change: 0 × MIC: $1.2 \pm 0.5$ and 0.1 × MIC: $0.28 \pm 0.3$; analysis of variance: $F=8.72$, d.f.=1, $P=0.042$; following the Geber method described in refs. [17,36]; Supplementary Fig. 7).

We confirmed this in additional experiments using two antibiotics with different modes of action at 0.1 × MIC (rifampicin, inhibition of RNA synthesis; tetracycline, inhibition of protein synthesis). Bacteria evolved anti-predator defence very rapidly with

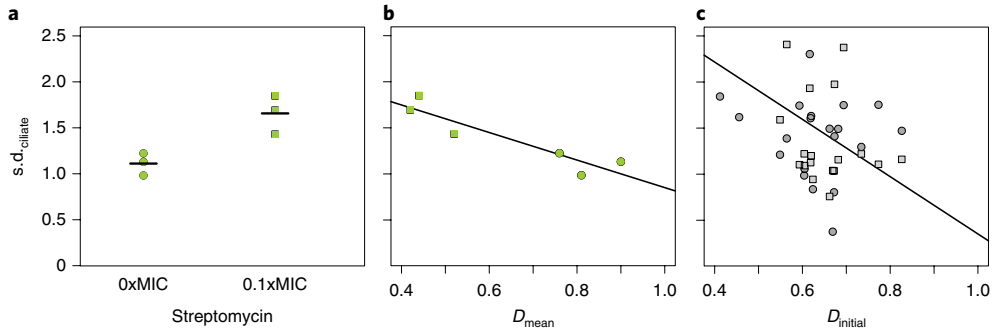


**Fig. 2 | Stability of bacteria–ciliate populations exposed to 0 × or 0.1 × MIC sub-MIC levels of streptomycin and the correlation of stability with defence levels of the bacteria populations. a**, Standard deviations (s.d. × $10^4$) for de-trended ciliate time series were significantly higher for populations exposed to 0.1 × MIC. Symbols, replicates; horizontal bar, mean (corresponding to Fig. 1b,d). **b**, Standard deviations were negatively correlated with the mean levels of defence that evolved over time; symbols correspond to **a**. **c**, Significant negative correlation between s.d. (s.d. for de-trended predator densities of the first ten transfers, that is before further evolution of defence) and initial defence level of the bacteria at the start of the experiment. Bacteria with different defence levels were grown in the absence of streptomycin (dark grey circles) and the presence of 0.1 × MIC streptomycin (light grey squares). For statistical tests, see main text.

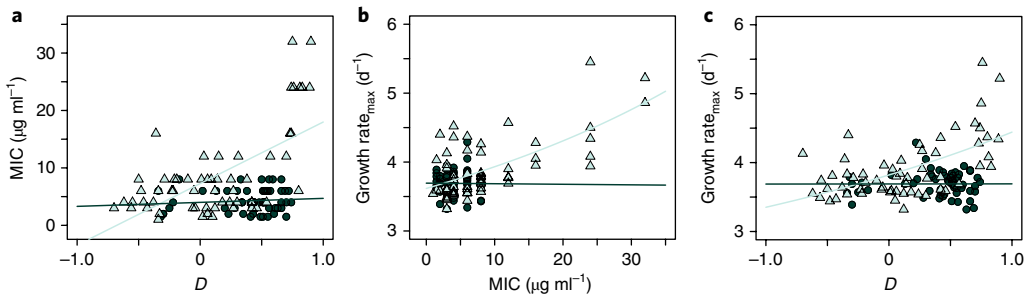


**Fig. 3 | Trait correlations of clonal isolates from *P. fluorescens* populations exposed to 0.1 × MIC streptomycin and the ciliate *T. thermophila* from the end of the experiment (day 66). a**, MIC and defence level *D*. **b**, Maximum growth rate of the bacteria and MIC (μg ml$^{-1}$). **c**, Maximum growth rate and *D*. Light grey triangles and regression lines represent clones from ciliates + 0.1 × MIC; dark grey circles and regression lines represent those from 0 × MIC and ciliates. For statistical tests, see main text.

tetracycline but not with rifampicin. Also in these cases bacterial population sizes were lower in the antibiotic treatment without predator, and defence evolution affected the stability of predator–prey dynamics with lower stability in the absence of defence evolution (Supplementary Fig. 8).

Our data show that the combination of sub-MIC levels of streptomycin and predation slowed down the evolution of anti-predator defence as well as antibiotic resistance. Clonal interference, differences in mutation supply, genomic constraints such as epistatic interactions and pleiotropic effects, and differences in the strength and directionality of selection could explain these observations[14,25,27,37–39]. In the case of clonal interference, we would predict to find subpopulations of clones that are either resistant against streptomycin or defended against ciliates, but not both. We did not find evidence for this when estimating correlations between these two traits in bacterial clones from populations with 0.1 × MIC streptomycin and ciliates isolated from the end of the experiment (Fig. 3a). We rather found a significant positive correlation indicating a pleiotropic effect for defence and resistance (GLM: log(MIC) ~ defence level: $F = 36.5$, d.f. = 1, $P = 1.2 \times 10^{-7}$; Supplementary Table 3). Such an effect was absent in the populations evolving in the presence of only the ciliates (GLM: log(MIC) ~ defence level: $F = 0.59$, d.f. = 1, $P = 0.44$; Fig. 3a). There were also no costs associated with defence and MIC levels of individual clones that could hinder the evolution of resistance or defence as we observed only positive correlations with maximum growth rates when testing in the absence of either of the stressors from the 0.1 × MIC streptomycin and ciliate populations (GLM: $r_{max}$~log(MIC): $F = 43.6$, d.f. = 1, $P = 1.5 \times 10^{-8}$; $r_{max}$~ defence level: $F = 30.7$, d.f. = 1, $P = 8.1 \times 10^{-7}$), which were absent in the one-stressor environments (Fig. 3b,c and Supplementary Table 3).

To further investigate the mechanisms slowing down evolution of defence and resistance, we used whole-genome sequence data from the replicate bacterial populations over time. For this, we isolated DNA from subsamples of the populations at ten time points (Methods; Supplementary Information). We applied a pipeline to distinguish mutations from sequence errors and identified CNVs, short variants (SNPs, INDELs) and cohorts of variants with similar dynamics over time. We found a large number of variants in all populations (Fig. 4a and Supplementary Fig. 9) probably because bacterial populations were not mutation-limited (average size > $10^8$ individuals), which also suggests that the role of drift was negligible in our populations. However, the number of variants differed significantly (GLM: $\chi^2 = 3393$, d.f. = 8, $P < 2.2 \times 10^{-16}$; Fig. 4a), with most mutations in the populations where bacteria evolved in the presence of 0.1 × MIC and fewest in the presence of the ciliate (Supplementary Table 4). These differences in the overall number of mutations are probably explained by the evolution of mutator lineages in some replicates (Fig. 4a). The majority of mutations were synonymous substitutions (Fig. 4b) and there were no differences

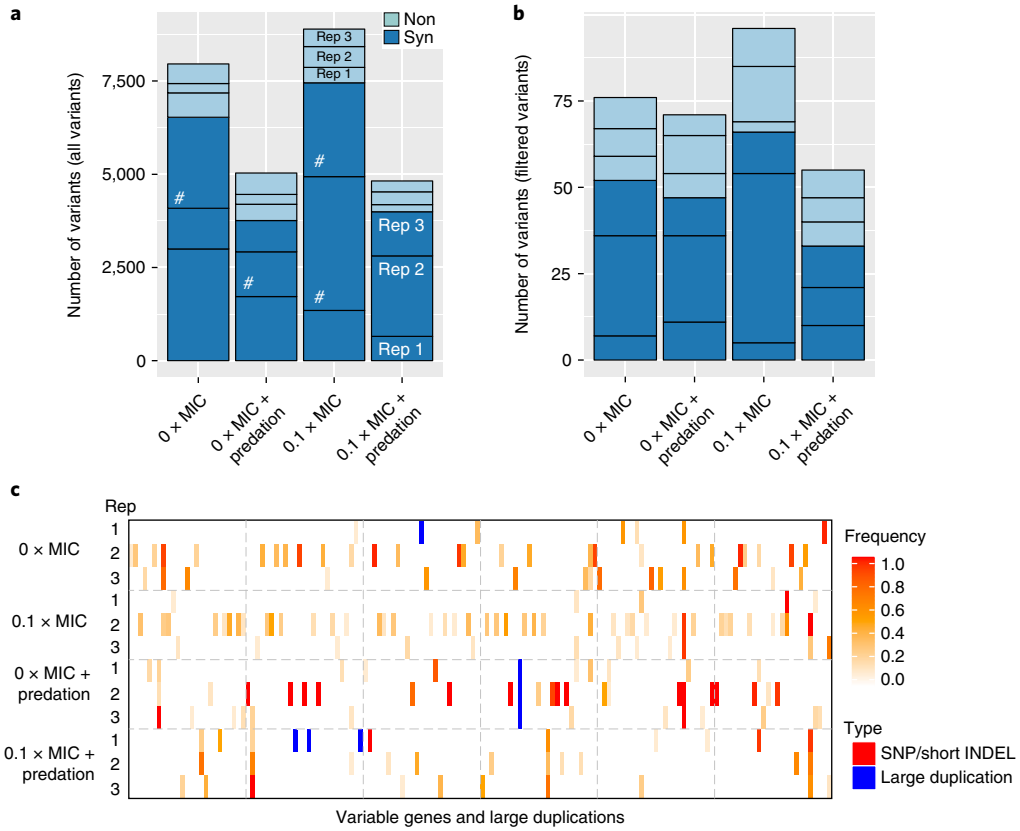


**Fig. 4 | Molecular evolution of *P. fluorescens* populations exposed to 0.1 × MIC streptomycin and the ciliate *T. thermophila* in a factorial design. a**, Total number of mutations (SNPs and small INDELs) accumulated over 66 days in *P. fluorescens* in the control populations, in the presence of predation, the presence of 0.1 × MIC streptomycin and in the presence of both. Blocks within the bars represent replicates (rep, $n = 3$). The hash symbol (#) represents the occurrence of a known mutator allele in the population (*mutL* or *mutS* gene). Non, non-synonymous; syn, synonymous. **b**, Number of mutations at high frequencies (>50%) in populations and in genes related to antibiotic resistance and anti-predator defence (see main text). **c**, Genomic variants across replicated populations for 145 genes and 5 large duplications in the *P. fluorescens* SBW25 genome. Only variants passing filtration criteria are displayed (total, 190 variants). Heat map colour from white (0.0) through orange (0.5) to red (1.0) indicates the maximum frequency of a SNP or short INDEL obtained in a population over time (66-day evolutionary experiment). Blue bars indicate the presence of large duplications. Columns represent variable genes or genomic duplications ordered from left to right according to their locus along the genome. Rows numbered 1–3 within treatments represent replicates 1–3.

between the treatments in the fraction of synonymous mutations (GLM with proportion data: $\chi^2 = 84.8$, d.f. = 8, $P = 0.33$), suggesting that there were no or only slight differences in mutation supply.

In further analyses, we focused on genes involved in antibiotic resistance and associated with the wrinkly spreader colony phenotype[40] and derived variants within genes that reached a frequency in at least one population of 50% (hereafter filtered variants). We focused on the wrinkly spreader phenotype as it has previously been shown to be selected by predation (the phenotype forms biofilm)[16] and we found a higher frequency of wrinkly spreaders in the presence of predation independent of the streptomycin concentration (GLM: predation: $F = 248.3$, d.f. = 1, $P = 2.627 \times 10^{-7}$; Supplementary Figs. 3 and 10 and Supplementary Table 5). The number of selected mutations differed significantly between the different treatments (GLM: $\chi^2 = 74.87$, d.f. = 8, $P = 0.0091$; Fig. 4b) with fewest variants in the environment with 0.1 × MIC and the ciliates present (GLM: predation: $\chi^2 = 79.24$, d.f. = 10, $P = 0.0076$; antibiotics: $\chi^2 = 79.18$, d.f. = 9, $P = 0.8168$; predation × antibiotics: $\chi^2 = 74.87$, d.f. = 8, $P = 0.038$).

We identified one gene related to antibiotic resistance (*rpsL*) in populations exposed to antibiotics alone and where we observed streptomycin resistance evolution (Figs. 1c and 4c). The third replicate, where we did not observe streptomycin resistance evolution, had no mutation in the known resistance related gene. For the populations exposed only to predation, we found in all three replicate populations a duplication that did not occur in other treatments arising around day 20–30 (Fig. 4c), and in two populations, the fixation of mutations in the gene PFLU 4745 (Figs. 1b and 4c). For populations evolving in the presence of antibiotics and the predator, we found different mutations reaching high frequencies. Mutations in the gene *ptsP* were found at high frequency in all three replicate populations, and mutations in *wspF* and *gacS* in two replicates. The latter two have previously been associated with the wrinkly spreader phenotype[41,42], and *ptsP* has a proposed global regulatory function for gene expression[43].

Mutations in the same genes in replicate populations can be considered evidence for fitness benefits of these mutations. While we found one to three multihit genes within treatments (Figs. 1 and 4), we found only one gene with derived variants present in the one- and two-stressor environments in at least two out of the three replicates (*ptsP*; Fig. 4c) but not in the control populations without any stressor. The lack of overlap across treatments but not across replicates within treatments in derived alleles suggests that different mutations were selected with one or two environmental stressors. Furthermore, in the one-stressor environment, derived alleles swept to high frequencies in all replicates (Fig. 4b,c). In the two-stressor environment, we found sweeps as well as additional sweeps at a later time point and before the preceding sweeps were close to fixation. In one replicate

(Fig. 4d, middle row, replicate 2) mutations that reached high frequencies (*ptsP* and *wspF*) went extinct and were replaced by others, which could either be the result of an additional detrimental mutation in this genetic background or clonal interference. Interestingly, these frequency changes correlate with changes in the predator–prey dynamics (Fig. 1d, top row, replicate 2; a decrease in predator and an increase in bacteria densities around day 50), which we did not observe in the other two replicates where the derived allele of *ptsP* stayed at high frequencies. We observed two or several derived alleles with the same trajectories within the same populations in the presence of 0.1 × MIC (Fig. 1c,d) indicating genetic hitchhiking, where driver mutations carry along other mutations.

Based on general ecological theory for predator–prey systems (Supplementary Fig. 1), we predicted differences in the eco-evolutionary dynamics of bacteria and ciliate communities in the presence and absence of antibiotic stress through slower evolution of anti-predator defences in the presence of the antibiotics. These differences in the evolutionary dynamics altered the ecological dynamics. Thus, our experiment showed a significant change in the link from evolution to ecology in the presence of both stressors. The slower phenotypic evolution was the result of different mutations increasing to high frequencies in the one- and two-stressor environments. Thus the genomic changes driving eco-evolutionary dynamics[11] and the link between evolution and ecology might depend on the system and the specific conditions.

For the two stressors examined here we can suggest that sub-MIC levels of antibiotics have significant ecological and evolutionary effects on communities, and alter the dynamics of the microbial loop as well as its link to ecosystem functioning and nature conservation[44]. Ours and other recent studies examining eco-evolutionary dynamics and multistressor selection[3,12,45] suggest that the type of species interaction and stressor determines the potential mechanism whereby multiple stressors affect the links between ecology and evolution. Consequently, the strength of the link between evolution and ecology depends on other stressors or environmental factors, making predictions on when to find eco-evolutionary dynamics challenging.

## Methods

**Study system and microcosm experiments.** As a prey species we used the bacterial strain *P. fluorescens* SBW25[46] and as a predator the ciliated protozoan *T. thermophila* 1630/1U (CCAP). Prior to the experiments, the bacterial stock was kept at −80 °C and ciliate stocks were cultured axenically in proteose peptone yeast (PPY) extract medium containing 20 g of proteose peptone and 2.5 g of yeast extract in 1 l of deionized water. All treatments were started from one clonal culture of bacteria to achieve minimum initial genetic variability in populations. Experiments lasted 66 days, representing approximately 220 bacterial and ciliate generations.

**Community experiments.** Experiments testing the community dynamics were conducted in standard 25 ml glass vials[12,17,40,47] with 6 ml of medium containing M9 salts and King's B nutrients at 5% concentration (5% King's B: 1 g $l^{-1}$ peptone number 3 and 0.5 ml $l^{-1}$ glycerol). Every 48 h, 1% of each culture was transferred to a new vial containing fresh culture medium. Microcosms were kept at 28 ± 0.1 °C and shaken constantly at 50 r.p.m. Population sizes were estimated using absorbance measurements and light microscopy counts[17]. Evolution of the prey defence trait *D* against predator grazing was quantified with a simple, ecologically appropriate bioassay where growth rates of the predator were measured and compared between ancestral and evolved prey[12,17]. We used Liofilchem MIC strips to measure antibiotic resistance over time for the evolving populations (Supplementary Information and Supplementary Fig. 5) and for clonal isolates from day 66. We set up a first experiment adding 0 × or 0.1 × MIC streptomycin to microcosms of bacteria with and without ciliates with three replicates per treatment (12 microcosms in total). A second set of experiments was set up at a later time point using 0.1 × MIC of rifampicin and tetracycline in bacterial microcosms with and without ciliates (4 replicates each, 16 microcosms in total). We analysed the second set of experiments separately. To assay colony phenotype frequencies, we plated diluted samples from day 66 on PPY agar and categorized the types according to ref. [40].

**Evolution of antibiotic resistance.** A second experiment was used to test for the interactive role of predation and a sub-MIC of streptomycin on the evolution of antibiotic resistance. The experiment was conducted in 96-well plates where populations were transferred into fresh culture medium every 48 h using a pin-replicator[48] in a medium without streptomycin or with 0.1 × MIC streptomycin, and with or without ciliates. Proportions of resistant populations were tested by plating each of the populations onto agar containing an above MIC concentration of streptomycin (25 μg $ml^{-1}$). For the analyses, we used the differences in the proportion of resistant populations between 0 and 0.1 × MIC per time point and contrasted these between the treatments with and without ciliate.

**Data analyses.** All statistical analyses were performed in the R statistical environment[49] using the *lme4*[50] and the *geepack*[51] packages. Data from the experiments with streptomycin and tetracycline/rifampicin were analysed separately as they were performed separately. We used consumer-specific GEE models (bacteria alone or bacteria and ciliate) for the analyses of bacterial and ciliate densities as well as predator–prey ratio and defence level *D* over time with day and sub-MIC (0 and 0.1 × MIC). We used the family Gamma and the link function inverse for density data and the family Poisson and the link function identity for *D*. For the stability analyses of the communities, we calculated the standard deviation of predator population size after de-trending the time series and scaling the mean to zero using the R package *pracma*[52]. To test for differences in stability between treatments and a relationship between stability and maximum *D*, we GLMs with the family Gamma and the link function inverse. Differences in ingestion rates for defended and naive bacteria (Supplementary Fig. 4) were tested using linear models. The evolution of resistance with and without ciliates in 48 replicate populations (Supplementary Fig. 6) was compared using GLMs with the family Gamma and the link function inverse. For the correlations between *D* and resistance of the clones from the end of the experiment, we used a GLM with the family Gaussian and the link function identity, and for the correlation between $r_{max}$ and *D* as well as $r_{max}$ and resistance we used GLMs with the family Gamma and the link function inverse. To test for the effect of predation on the frequency of wrinkly spreader evolution, we used GLMs with the family Gamma and the link function inverse.

**Sequence analyses.** Bacterial DNA was extracted (DNeasy Blood & Tissue Kit, Qiagen) directly from 0.5 ml freeze-stored whole-population samples without culturing steps to retain allele frequencies intact. We sequenced the following populations: (1) populations without antibiotics or predators (control); (2) with antibiotics (0.1 × MIC streptomycin); (3) with predators; and (4) with both predators and 0.1 × MIC streptomycin. For each treatment, all three replicate populations were sequenced from 10 time points over the course of the 66-day experiment. We focused on early time points, since adaptive mutations were expected to emerge early in rapidly evolving bacterial populations (sequence data generated for days 2, 4, 8, 12, 22, 32, 42, 50, 56, 66). Paired-end libraries were prepared using Illumina Nextera XT sequence reads obtained by high-throughput sequencing (Illumina Nextseq 500 high output; for coverage see Supplementary Table 6).

After mapping reads to the reference genome (*P. fluorescens* SBW25 NC_012660)[53], variants (SNPs and short INDELs) were called using HaplotypeCaller and jointly genotyped for all 10 time points per population using GenotypeGVCFs with GATK (version 3.5) and ploidy set to 30. Thus, for each population, we could detect variants at each locus at a frequency detection limit and resolution of 3.3% (100/30%). Variants were hard-filtered to omit variants with combined read depth < 100 and Phred-scaled quality < 50. We used SnpEff[54] with the annotation file corresponding to the reference genome for variant effect prediction, that is, to detect whether the variant has no predicted effects (non-coding variants, intergenic regions and synonymous variants) or results in an amino acid change (all coding, non-synonymous variants). Prior to further analyses, variant counts (maximum 30) were converted into frequencies (0–1).

We designed a pipeline to remove probable sequence errors from the resulting dataset utilizing previously published pipelines[25,55] (see also Supplementary Information). To reliably track variant frequency, we excluded variant loci represented by two or more alternate alleles in the same population in GATK variant calling. Since the frequency of a real mutation is expected to be correlated across time points, we excluded variants whose frequency trajectories had a lag-1 autocorrelation of <0.2. Variants occurring immediately at detectable frequency are more likely to be either ancestral variants or sequence errors compared to variants emerging at later time points. Therefore, initial variants (the first two sequenced time points) were required to have a stricter minimum lag-1 autocorrelation of 0.5. Because variants that remain at very low frequencies are unreliable, we required a variant to reach a frequency of 0.1 in a minimum of two time points. We also excluded variants located within 10 base pairs from INDELs, which might have an increased likelihood of being alignment errors. Finally, to ensure that the data have sufficient temporal resolution, we removed variants with missing information from over two (>2/10) time points (resulting, for example, from insufficient coverage at the variant locus in a given sample).

As well as analysing mutations individually, we assigned them to cohorts, that is temporal clusters of mutations, using a previously developed approach[25]. First, a Euclidian distance matrix was created from frequency vectors of mutations with ≥0.3 maximum frequency, since low-frequency mutations cannot be reliably

clustered. The distance matrix was hierarchically clustered, and the hierarchies were flattened using a cutoff distance of 0.5 (data resolution did not permit lower cutoff distances), using the dist, hclust and cutree functions in the stat package in base R. After all filtration steps, we also extracted non-synonymous candidate mutations potentially under selection based on the variant being located on a gene mutated at a minimum of 50% frequency in at least one non-mutator population (to leave out non-selected hitchhikers probably present in the mutational cohorts of mutator populations).

In addition to the detection of SNPs and short INDELs using the approach outlined above, we performed read-depth-based detection of large genomic deletions and duplications (that is, copy number variation, CNV) using cnvnator 0.3.2[56] with a bin size of 500 base pairs. CNVs of interest were extracted based on absence in the first sequenced time point (probable ancestral CNV or sequence error) and detection in at least two consecutive time points (signal of potential selection).

To test for differences in the total number of variants and the number of filtered variants between treatments, we use generalized linear models with the family Poisson and the link function log with treatment as factor. We used GLMs with the family Gamma and the link function inverse to test for the distribution of variants (total and filtered) in different impact classes with the presence and absence of streptomycin, predators and impact class as factors.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Data availability

Data reported in the paper will be archived in a community archive. Raw sequence reads from genomic analyses have been deposited in the NCBI Sequence Read Archive under the BioProject accession number PRJNA476204. Count and trait data have been deposited at PANGAEA: https://doi.pangaea.de/10.1594/PANGAEA.895614.

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## Author contributions

T.H., M.J. and L.B. conceived and designed the study. J.C., J.F., E.K. and L.B. analysed the sequence data. S.K. performed the sequencing. T.H. and J.C. collected the data. L.B. and T.H. analysed the data. J.F., T.H. and L.B. wrote the manuscript. All authors contributed to the final version of the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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