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Resource abundance and the critical transition to cooperation

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Abstract

Cooperation is abundant in nature, occurring at all levels of biological complexity. Yet cooperation is continually threatened by subversion from non-cooperating *cheaters*. Previous studies have shown that cooperation can nevertheless be maintained when the benefits that cooperation provides to relatives outweigh the associated costs. These fitness costs and benefits are not fixed properties, but can be affected by the environment in which populations reside. Here, we describe how one environmental factor, resource abundance, decisively affects the evolution of cooperative public goods production in two independent evolving systems. In the Avida digital evolution platform, populations evolved in environments with different levels of a required resource, whereas populations of *Vibrio cholerae* evolved in the presence of different nutrient concentrations. In both systems, cooperators and cheaters co-existed stably in resource-rich environments, whereas cheaters dominated in resource-poor environments. These two outcomes were separated by a sharp transition that occurred at a critical level of resource. These results offer new insights into how the environment affects the evolution of cooperation and highlight the challenges that populations of cooperators face when they experience environmental change.

Introduction

Cooperation takes many forms in nature, and each instance presents an evolutionary opportunity for exploitation by those that take advantage of the costly behaviour but do not contribute. Over time, these *cheaters* can drive cooperators from the population and lead to a tragedy of the commons, whereby the entire population declines (Hardin, 1968). How cooperators persist despite these formidable challenges is often attributed to inclusive fitness (Hamilton, 1964a,b), where an individual's fitness is determined not only by its own activities, but also by the effects that cooperation has on its relatives. With this perspective, cooperative behaviours can flourish when the benefits they provide to close relatives outweigh the associated costs (West *et al.*, 2007).

Quantifying these fitness costs and benefits is often quite difficult. Compounding this difficulty is the fact that evolution does not occur in a vacuum. In natural settings, a myriad of biotic and abiotic factors combines to continually alter the costs and benefits of a cooperative act as well as the relatedness of the individuals involved. These fluctuations in fitness effects over both time and space make the maintenance of cooperation over evolutionary timescales even more daunting.

Here, we examine how one such environmental factor, resource abundance, affects the evolution of biofilm formation, a prevalent form of cooperation within microbial communities (Hall-Stoodley *et al.*, 2004). Biofilms are spatially structured communities of microorganisms connected through a matrix of extracellular polymeric substance (EPS), which is produced by individuals at a cost and placed into the surrounding environment. The biofilm lifestyle often affords its constituents a wide variety of benefits, including adhesion to surfaces in fluid environments, protection from predation and increased resistance to antibiotic

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treatments (Mah & O'Toole, 2001; Mah *et al.*, 2003; Hall-Stoodley & Stoodley, 2009). As EPS is a *public good* that can be utilized by the surrounding community, biofilms present an opportunity for exploitation by cheaters, which take advantage of the biofilm without contributing to its formation (Xavier & Foster, 2007; Brockhurst *et al.*, 2008). The presence of cheaters can significantly weaken biofilms, thereby reducing the potential benefits and threatening the long-term stability of cooperation (Rainey & Rainey, 2003; Popat *et al.*, 2012).

In this article, we employ two experimental systems, one digital and the other biological, to investigate how resource abundance affects the evolution of cooperative biofilm formation. First, using the Avida digital evolution platform (Ofria & Wilke, 2004), populations of 'digital organisms' evolved in spatially structured environments containing different levels of a resource. This resource was required to complete logic-based tasks that conferred fitness rewards. One task, however, yielded no direct reward when completed, but instead produced a diffusible public good. Sufficient levels of this public good allowed patches to survive a periodic environmental disturbance.

To further explore the relationship between resource abundance and cooperation in a living system, we performed additional experiments in which populations of the bacterium *Vibrio cholerae* grew and evolved in environments with different nutrient concentrations. In these experiments, the formation of biofilms enhanced survival of periodic population bottlenecks. The amount of biofilm produced and the composition of each population were measured to determine the success of evolved cooperators in each environment.

Although the microbial and digital systems used in this study were quite distinct, we observed the same evolutionary response to resource abundance. Specifically, we found that cooperation does not simply increase as resource becomes more abundant and, rather, that it can be maintained only above a critical level of resource.

Materials and methods

Computational model

Avida (Ofria & Wilke, 2004) is a computational platform that has been used to study complex ecological and evolutionary dynamics in a wide variety of contexts (see, e.g. Wilke *et al.*, 2001; Lenski *et al.*, 2003; Chow *et al.*, 2004; Clune *et al.*, 2012). This section provides a brief overview of Avida and describes how it was used to model the cooperative formation of biofilms.

Experiments in Avida act on populations of self-replicating digital organisms (small computer programs), each of which resides independently in a cell on a lattice. As depicted in Fig. 1, each organism is 'embodied' by a circular list of instructions (its *genome*), which are executed on virtual computer hardware allocated to that organism. This virtual hardware includes registers and stacks for storing data values and a central processing unit (CPU) for executing instructions. Instructions perform arithmetic and logical operations (e.g. addition, multiplication and bit-shifts), control the flow of execution, perform input and output and execute behaviours associated with replication. Instructions are typically executed sequentially, but some instructions enable execution to jump to a different location in the genome. An organism's environment includes a small set of random numbers that can be input and stored in registers; an organism can also output the value of a register to the environment.

In this study, populations were seeded using a single ancestor organism capable only of replication. Any additional behaviours, including the completion of logic tasks described below, arose through mutations that occurred during asexual replication. An organism replicated by first allocating additional space in its genome, into which it copied instructions from its genome line by line. During this process, mutations substituted one instruction with another with probability 0.75%. Mutations also deleted or inserted a randomly chosen instruction from the set of 26 instructions with probability 5% as the organism divided. Once this process completed, the genome was cleaved, and the resulting daughter organism was placed into a randomly chosen adjacent cell, replacing any organism occupying that cell. This process created positive assortment of types. As genomes are circular, an organism continued to execute until it was replaced, typically replicating with each pass through the genome.

Individuals competed for space in a 100×100 cell lattice by completing a set of binary logic *tasks*, listed in Table 1. Whenever an organism output a number to its environment, Avida determined whether that value matched the result of any of the logic tasks when applied to numbers in the organism's initial environment. If so, Avida recorded that the organism had completed the task and conferred on the organism a *merit* bonus proportional to that task's complexity (for a discussion of task complexity, see Lenski *et al.*, 2003). These merit bonuses, awarded once for each unique task completed, were given to both the parent and offspring immediately following replication. During each *update*, Avida's unit of time, each organism executed an average of 30 instructions. An organism with more merit than its competitors executed proportionately more instructions from its genome per update. Therefore, by completing these tasks, an organism replicated more quickly, thus increasing its fitness.

In addition to random numbers, the Avida environment also contains *resources*. In this study, populations evolved in environments with different levels of a

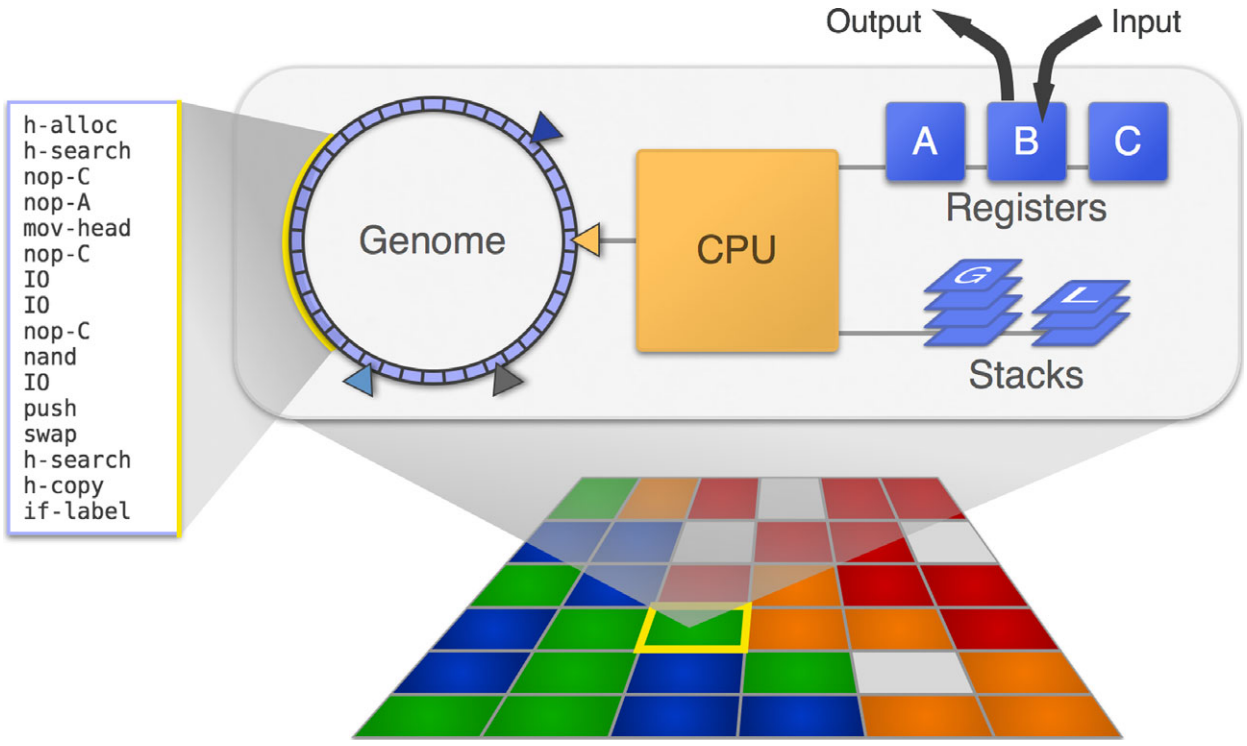

Fig. 1 Anatomy of an Avidian. Within populations (*below*), each individual organism (*top*) possesses a circular genome of instructions, which are executed on 'virtual computer hardware' to produce that individual's phenotype. Organisms compete for resources and space in their environment by completing logic tasks and can produce and excrete products into their surrounding environment.

Table 1 Binary logic tasks in the Avida biofilm model.

Task	Logic operation	Output*	Merit bonus
NOT	$\neg A$, $\neg B$	0110, 0101	2
NAND	$\neg(A \wedge B)$	0111	2
AND	$A \wedge B$	1000	4
OR NOT	$A \vee \neg B$, $\neg A \vee B$	1101, 1110	0†
OR	$A \vee B$	1011	8
AND NOT	$A \wedge \neg B$, $\neg A \wedge B$	0001, 0010	8
NOR	$\neg(A \vee B)$	0100	16
XOR	$(A \wedge \neg B) \vee (\neg A \wedge B)$	0011	16
EQU	$(A \wedge B) \vee (\neg A \wedge \neg B)$	1100	32

The symbol ¬ denotes negation.
*Outputs are listed for the example input values *A*: 1001, *B*: 1010. Note that Avida typically uses 32-bit integers.
†The cooperative OR NOT task did not confer a merit bonus.

required resource R, which diffused throughout the lattice. One unit of R was required to complete any task. As described by eqn 1, the level of R was determined for each cell c at the beginning of each update t, where R_I represents the total inflow of resource, C is the set of all lattice cells, N_c is the set of cells adjacent to cell c, φ is the rate of resource diffusion into neighbouring cells and δ is the rate of resource decay. For all experiments reported, $|C| = 10000$, $\varphi = 0.01$, and $\delta = 0.01$. The inflow rate R_I and initial resource level $R_c(0)$ were adjusted to create environments with equilibrium R levels ranging from 2 to 64 units per cell.

$$R_c(t+1) = \frac{R_I}{|C|} + (1-\varphi)(1-\delta)R_c(t) + \sum_{n \in N_c} \frac{\varphi(1-\delta)R_n(t)}{|N_c|} \quad (1)$$

The environment was defined by eight binary logic tasks (Table 1), each of which was rewarded proportionally to its complexity. A ninth task of intermediate complexity was defined that did not confer a merit reward. Instead, the completion of this OR NOT task created one unit of a public good P, which was placed into the environment at that location. P also diffused and decayed at a rate of 1% per update. This public good, akin to EPS produced by biofilm-forming bacteria, enabled organisms to survive a periodic environmental disturbance. During each update, this disturbance event randomly selected one lattice cell and examined each neighbouring cell within a 5-cell distance, a region totalling 121 cells. If the average level of P in these cells was below three units, all organisms residing in that region were killed. Otherwise, all organisms were spared. These environmental

disturbances presented an opportunity for nonproducers to exploit the public goods produced by neighbouring cooperators. Importantly, the formation of these 'biofilms' did not affect the flow of *R* or *P* in the environment (see Nadell *et al.*, 2010 and Julou *et al.*, 2013 for studies where flow *is* affected, leading to different biofilm structures). Individuals could not directly sense the level of resource in their surrounding or the presence or phenotypes of neighbouring individuals.

For each of the equilibrium *R* levels studied, 30 replicate populations evolved for 100 000 updates, or an average of 16 700 generations. Each simulation started with a different seed for the pseudorandom number generator, which allowed each population to follow a unique evolutionary trajectory.

To allow populations to first reach sufficient densities, the environmental disturbance began at update 1000. Otherwise, populations tended to die out. However, we note that the formation of biofilms was not necessary for survival. Most populations persisted throughout the duration of the simulations, regardless of the level of *R*. By forming biofilms, however, populations could maintain greater densities.

In all resource environments, the periodic disturbances drove a subset of the replicate populations to extinction. This typically occurred early in the simulations, when populations had not yet reached sufficient densities. We found no relationship between resource abundance and the proportion of populations that met extinction. Data for populations driven to extinction are not included in our results.

Ancestral strain and growth media

Our wild-type strain of *Vibrio cholerae* was derived from the wild-type El Tor biotype strain C6706str2, a streptomycin-resistant isolate of C6706 (Thelin & Taylor, 1996). Cultures were grown in Miller LB broth (Neogen Corp. Acumedia Product #7279, Lansing, MI, USA). To provide different resource environments, LB concentrations of 0.25×, 0.5×, 0.75×, 1×, 1.25×, 1.5×, 1.75× and 2× were created by serially diluting a 2× LB stock with a 2× NaCl solution (20 g L^{-1}), thereby ensuring an equivalent salt concentration at each resource level.

Experimental evolution of biofilm formation

To start cultures, cells were placed from frozen glycerol stock into 2 mL 1× LB and incubated overnight with vigorous (220 rpm) shaking at 35 °C. Cultures were then diluted 100-fold in fresh medium for each LB concentration studied and vortexed, and 160-μL aliquots were placed into wells in a sterile MBEC assay plate (Innovotech Inc.; Harrison *et al.*, 2005). For each concentration, five replicate populations were grown for 24 h at 35 °C on a fixed-speed nutating mixer (Fisher Scientific Inc. Model 22-363-152). To remove cells from the resulting biofilms, the lid was placed into a sterile microtitre plate containing 150 μL 1× LB per well and sonicated on a suspended tray for 45 min at 40 kHz (Bransonic 2510 bath sonicator, Branson Ultrasonics Corp., Danbury, CT, USA). The resulting product in each well was then diluted 100-fold using the appropriate media, and 150 μL was used to inoculate the same well in a sterile MBEC plate. The remainder was mixed with 30 μL 80% glycerol per well and stored at −80 °C. This process was repeated for 7 days.

Biofilm growth measurement

Biofilms were measured by crystal violet staining. Cultures were started and grown as previously described; 5 μL samples were then vortexed with 145 μL of the appropriate media concentration and used to inoculate wells in an MBEC assay plate. After 12 h of growth in conditions identical to those described above, plates were washed with 150 μL phosphate-buffered saline (PBS), fixed in 150 μL 95% ethanol (EtOH) and stained with 150 μL of 0.41% crystal violet solution (w/v in 12% EtOH). Following three additional washes with 150 μL PBS, the crystal violet was diluted in 300 μL 95% EtOH. The absorbance of the diluted crystal violet was then measured via spectroscopy at 595 nm (SpectraMax M5, Molecular Devices, LLC, Sunnyvale, CA, USA).

Estimating population composition

In *V. cholerae*, biofilm formers display a *rugose* colony morphology, whereas nonformers display a *smooth* morphology when plated on LB agar (see Fig. 6 inset; Yildiz *et al.*, 2004). To estimate the proportions of cooperative biofilm formers and nonforming cheaters present in populations, we compared the abundances of these two morphotypes. Biofilms were first removed from the pegs on which they were grown by sonication, as previously described. The resulting solutions were serially diluted in 1× LB, plated on LB agar and incubated at 35 °C. After 24 h, the number of rugose and smooth colony-forming units (CFUs) was counted by visual examination.

Fitness of evolved strains in biofilm and planktonic growth

One evolved rugose type and one evolved smooth type were isolated from one replicate evolved population (1× LB) and frozen as previously described. To compare these isolates, cultures were grown overnight in 1xLB. Competitions were initiated by combining equal volumes of the two overnight cultures after dilution to equal densities (absorbances at 595 nm). Rugose, smooth and mixed cultures were grown in 1× LB overnight at 35 °C in an MBEC assay plate. Biofilms were

sonicated from the pegs into 0.86% saline, diluted and plated on LB agar. For planktonic growth, the cultures were extracted from the wells, diluted and plated on LB agar. Rugose and smooth CFUs were then counted after incubation. These experiments were repeated using absorbance at 595 nm as a proxy for density. We used the change in frequency of rugose types during growth as a measure of its relative fitness (v). Specifically, $v = p_{24}(1 - p_0)/p_0(1 - p_{24})$, where p_0 is the initial proportion of rugose CFUs per mL, and p_{24} is their proportion after 24 h (Ross-Gillespie *et al.*, 2007).

Software, data analysis and archival

Avida 2.12.4 was used for the simulations described. Data analysis was performed using R 3.3.2 (R Core Team, 2016). Reported confidence intervals were estimated by bootstrapping with 1000 resamples. The model, configurations, data and analysis scripts are available online (Connelly *et al.*, 2017).

Results

In the Avida model, populations were grown and evolved in environments with different levels of a resource. Although this resource was not necessary for growth, it was required for completing self-benefitting tasks. This resource was also required to complete a cooperative task that produced a public good. As described above, a periodic environmental disturbance killed all organisms in a localized region when the amount of this public good present in that region was below a configured threshold. In this study, *cooperators* are defined as individuals that completed the public good-producing task at least once during their lifetime, whereas *cheaters* did not. This classification is irrespective of the self-benefitting tasks performed, so many different cooperator and cheater phenotypes were possible. Cheaters could increase their survival rates under the protection of neighbouring cooperators.

Cooperation is maintained above a critical resource threshold

The relative frequency of cooperators at the end of simulations depended on the abundance of resource in the environments in which populations evolved (Fig. 2a). In *resource-rich* environments (i.e. those with 20 units of resource per cell and greater), cooperators uniformly represented 46.8% (95% CI [44.2%, 49.6%]) of their populations (Fig. 2a; see also Fig. S2). Conversely, cooperator frequency was significantly lower in environments with 14 units of resource per cell or less. In these *resource-poor* environments, individuals that completed the cooperative task were either absent or exceedingly rare (approx. 2.9%; 95% CI [2.09%, 4.03%]). Importantly, the transition between these two regimes occurred rapidly at intermediate levels of resource (approx. 14–20 units per cell).

As a result of increased cooperator abundances, populations in resource-rich environments produced more public good than populations in resource-poor environments (Fig. 2b). In fact, the average per cell level of public good produced by these evolved populations was substantially higher than the amount required to survive the environmental disturbance. As shown in Fig. 2c, this behaviour enabled populations in resource-rich environments to survive approximately 70% (95% CI [66.2%, 72.5%]) of the environmental disturbance events, whereas the survival rate was only 3% (95% CI [1.2%, 5.1%]) among populations in resource-poor environments.

In our model, localized reproduction and environmental disturbances created positive assortment, which can promote cooperation (Kerr *et al.*, 2002; Pepper & Smuts, 2002). As depicted in Fig. S1, these processes segregated cooperators and cheaters into different patches. Although these changes continually altered the spatial distribution of phenotypes (Video S1), the overall abundances of the cooperator and cheater phenotypes reached stability (Figs. 3, S2). In the population shown in Fig. 3b, for example, cooperators reached a frequency of approximately 45% shortly after environmental disturbances had nearly driven that population to extinction at around update 35 000.

Populations with cooperators reach higher densities

Populations in resource-rich environments maintained greater densities than those in resource-poor environments (Fig. 2d). These increases in population size were not simply due to the addition of resource. In control simulations where completion of the cooperative task did not produce public good, population sizes were not significantly affected by resource level (Fig. 2d). Therefore, these larger populations were not the result of environmental enrichment, but rather the increased survival of environmental disturbances provided by the cooperative production of public good (Fig. 2b,c). This finding indicates that public good production provided a selective advantage in resource-rich environments.

Cooperator success is not due to increased mutational opportunities

Importantly, because populations in resource-rich environments were larger, they experienced more growth, and therefore more mutational opportunities. Because of this, the relative lack of cooperation in resource-poor environments seen in Fig. 2a may have occurred because these populations had not yet evolved to complete the cooperative task. However, Fig. S3a shows that the cooperative task arose in all environments and that the rate at which populations acquired this

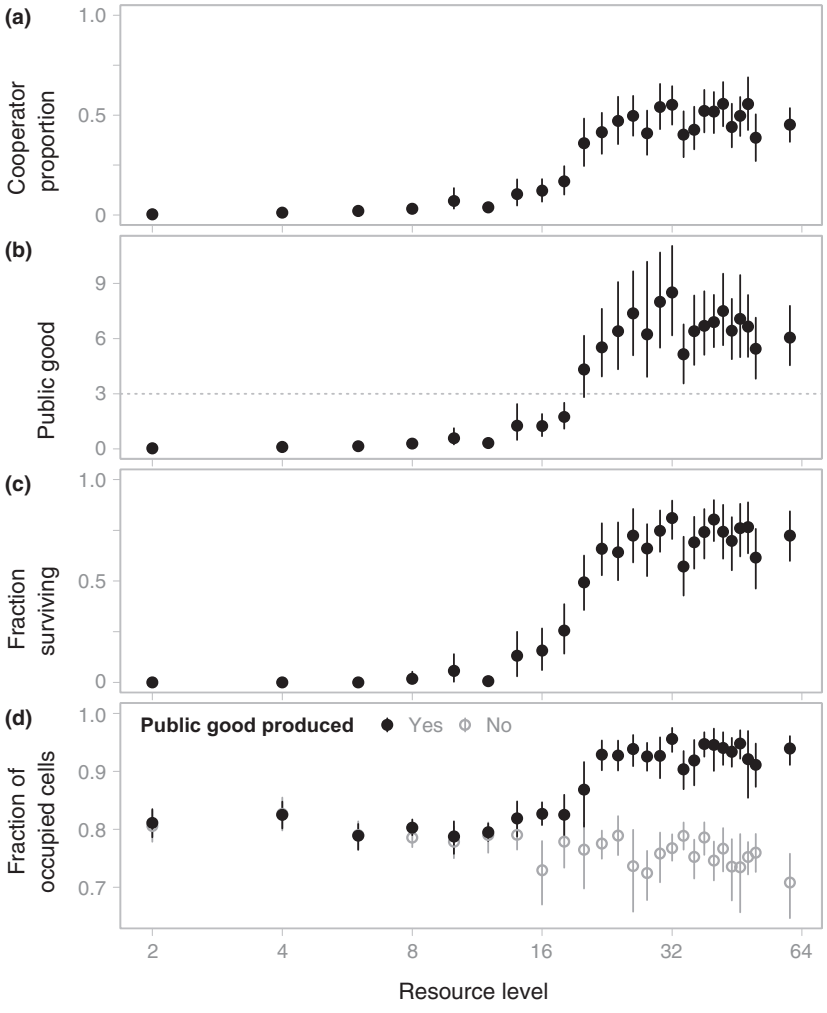

Fig. 2 Cooperation and survival at different resource levels. (a) Although cooperation arose in all environments, it was only maintained at high proportions when resource was abundant. (b) In environments where cooperation was maintained, the average level of public good exceeded the threshold for survival imposed by the environmental disturbance events (three units, *dotted line*). (c) These high levels of public good in resource-rich environments enabled populations to survive the majority of environmental disturbances. (d) As a result, populations in resource-rich environments maintained greater densities. When the cooperative task did not produce public good (*open grey points*), density did not change with resource abundance. For all panels, data shown are the averages among replicate populations at the end of simulations, and disturbance data are from the final 100 updates. Error bars indicate 95% confidence intervals.

behaviour was not affected by resource level. In fact, populations evolved to complete the most complex (and most beneficial) task, EQU, in all environments (Fig. S3a). Although the time required for this trait to first arise was not significantly affected by resource level (Fig. S3b), the number of populations in which it arose was (Fig. S3a).

Changing environments affect cooperation

We next subjected populations to changes in resource levels that crossed the critical threshold. Starting in either resource-rich ($R = 40$) or resource-poor ($R = 10$) environments, populations evolved for 30 000 updates before the resource level abruptly changed. During the next 30 000 updates, populations from the resource-rich environment experienced the resource-poor environment and vice versa. A second environmental shift then brought resource levels back to their initial values for the remainder of the simulations.

These environmental shifts strongly affected cooperation in both treatments (Fig. 4). When populations transitioned from resource-poor to resource-rich environments, cooperation increased substantially. Conversely, when resource levels shifted from rich to poor, cooperation quickly dropped. These results demonstrate that environmental changes can rapidly alter the types of interactions that occur within populations.

Critical resource threshold in *Vibrio cholerae*

To determine whether this critical transition to cooperation is a general feature of the evolution of cooperation in different resource environments, we also conducted experiments using a microbial system. Populations of the bacterial pathogen *Vibrio cholerae* were grown in different concentrations of LB medium in MBEC assay plates (Harrison *et al.*, 2005), where the formation of biofilms allowed constituent cells to adhere to polystyrene pegs protruding from the lid into each well. Periodically, biofilms were sonicated from the pegs, diluted

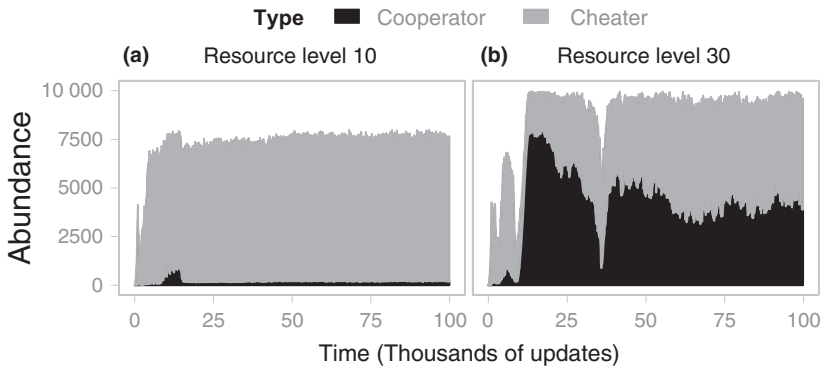

Fig. 3 Population dynamics of cooperation in different environments. The number of cooperators and cheaters are shown over time in two representative populations. Note that cheater abundances are shown stacked on top of cooperator abundances. (a) In a resource-poor environment, cheaters maintain their initial dominance throughout the simulation. (b) In a resource-rich environment, cooperators quickly rise in abundance immediately after their population is drastically thinned by environmental disturbance. Cheaters nearly drive cooperators to extinction by approximately 35 000 updates. However, the susceptible population was thinned, which once again allowed cooperators to rebound.

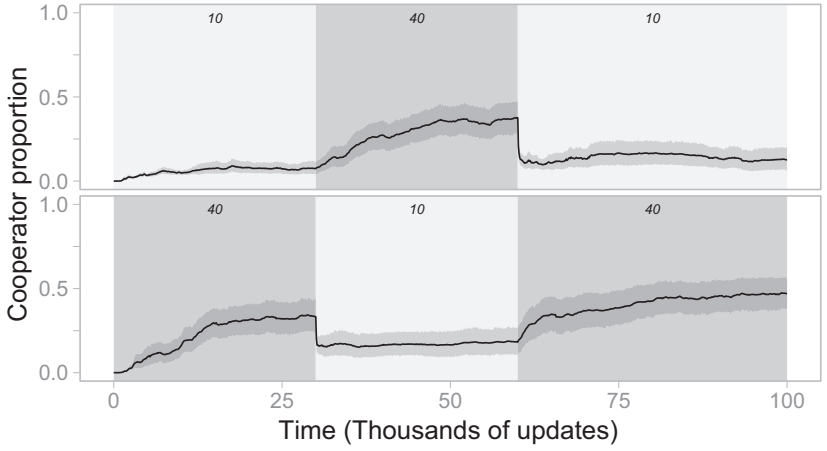

Fig. 4 Cooperation in changing environments. Evolving populations experienced an environmental shift at updates 30 000 and 60 000. Numbers above each region indicate the per cell equilibrium resource level (R). Mean cooperator proportion is shown among replicate populations, whereas shaded areas indicate 95% confidence bands. (top) When populations shifted from resource-poor to resource-rich environments, mean cooperator proportion increased from 7% to 38%. Upon returning to a resource-poor environment, mean cooperator proportion rapidly fell and remained near 12%. (bottom) Similarly, when populations temporarily experienced resource-poor environments, mean cooperator proportion dropped from 33% to 17%. When populations were returned to resource-rich environments, mean cooperator proportion reached 47%.

and used to inoculate a fresh plate. This process was repeated for 7 days. Importantly, the wild-type, ancestral strain exhibits poor biofilm formation due to repression of genes that synthesize EPS by the density-dependent process of quorum sensing (Hammer & Bassler, 2003; Zhu & Mekalanos, 2003; Waters *et al.*, 2008).

Populations that evolved in resource-rich environments formed significantly more robust biofilms than those that evolved in resource-poor environments (Fig. 5). As with the simulated populations, this increase in cooperation occurred rapidly above a critical level of resource. In the environments studied, this transition occurred between 0.5× and 0.75× LB. We refer to environments with levels of resource below this threshold as resource-poor (0.25× and 0.50× LB) and those above it as resource rich (0.75× LB and greater). Although EPS production was enriched in all environments due to our method of daily passaging, the increases seen in resource-rich environments were significantly greater than those in resource-poor environments (Fig. 5). Among the environments *within* each of these two regimes, no significant differences in biofilm formation were observed, again leading to a bimodal distribution of noncooperators and cooperators. In contrast, biofilm formation by the ancestral wild-type strain was not affected by resource concentration (Fig. 5).

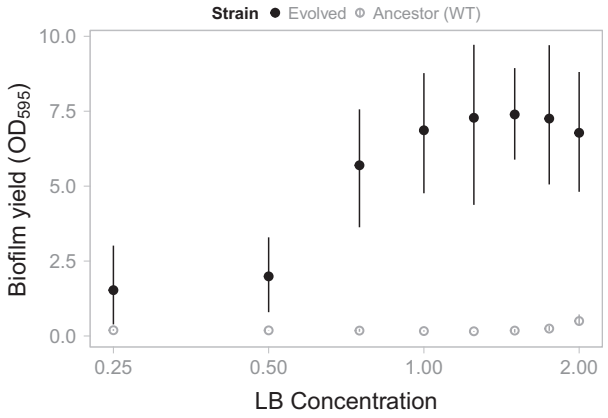

Fig. 5 Biofilm formation in different resource concentrations. Biofilm formation increased among all evolved strains (*filled black points*). However, strains that evolved in resource-rich environments (LB concentrations of approx. 0.75× and above) formed substantially larger biofilms than those that evolved in resource-poor environments (LB concentrations below 0.75×). The wild-type ancestral strain formed weak biofilms in all environments (*open grey points*). Error bars indicate 95% confidence intervals.

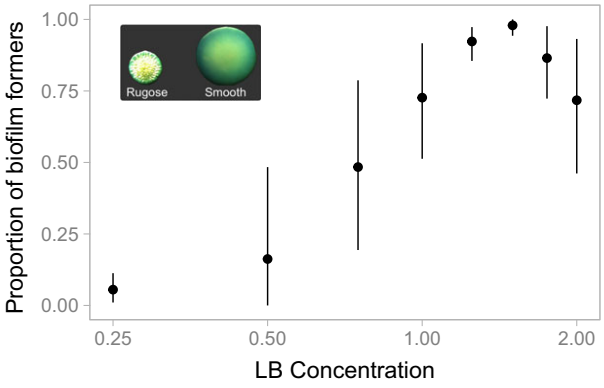

Fig. 6 Relative frequency of biofilm formers increases with resource richness. The average proportion of biofilm-forming types within five replicate populations is shown with the LB concentrations in which they evolved. Evolved populations were plated, and the number of biofilm-forming *rugose*- and nonforming *smooth* colony-forming units (CFUs) was counted. Error bars indicate 95% confidence intervals. Inset: Rugose and smooth colony morphologies exhibited by *V. cholerae*. Images are to scale.

This indicates that the increases in biofilm formation by evolved populations were not simply due to increased access to resource or nutrient regulation of biofilm formation. Moreover, the lack of biofilm formation at low resource levels was not due to a lack of growth, as *V. cholerae* was able to reach densities in 0.5× LB equivalent to those observed at 1.75× and higher than those at 2.0× (Fig. S4). Therefore, the differences in biofilm formation among evolved populations are not due to adaptive responses to different environments as has been observed in other species (Vivas *et al.*, 2008; Ramli *et al.*, 2013), but rather to selection for increased EPS production.

EPS production by *V. cholerae* leads to altered colony morphology (Yildiz *et al.*, 2004). As depicted in Fig. 6 (inset), *smooth* colonies, which are larger and appear more translucent, form poor biofilms, whereas *rugose* colonies, which are smaller in size and wrinkly in appearance, form robust biofilms. Consistent with its poor biofilm formation, the wild-type ancestor strain displayed a smooth colony morphology. Figure 6 shows that the proportion of biofilm-forming rugose types increases in resource-rich environments, which corresponds with the observed increases in biofilm formation (Fig. 5).

Non-EPS producers utilize public goods production to increase fitness

Biofilm formation in *V. cholerae* has been shown to be a cooperative trait allowing sequestration of public goods (Drescher *et al.*, 2014), while providing mechanisms to exclude conspecific members from the group (Nadell *et al.*, 2015). We therefore sought to determine whether biofilm formation in our experimental evolution studies was exploitable. Six unique smooth and rugose clones were isolated from the 1× evolved lines. As expected, EPS production by the rugose isolates significantly enhanced biofilm formation vs. smooth cells (Fig. 7a). When rugose and smooth cells were co-inoculated at approximately equivalent initial frequencies, smooth isolates dominated the planktonic culture (Fig. 7b). After growth in monoculture for 24 h, approximately 10^4 smooth cells were measured on the pegs as compared with approximately 10^6 rugose cells (Fig. 7c). In mixed cultures, the number of rugose cells associated with the pegs did not significantly change; however, the number of smooth cells increased more than 100-fold to 2×10^6 cells, nearly the same as the number of rugose cells. Importantly, however, on average 50% of the biofilm consisted of smooth cells (95% CI [36.2%, 57.5%]), indicating these cells were able to access the biofilm formed by rugose cells and survive the passaging event. Therefore, EPS produced by rugose cells could serve as a cooperative public good that was utilized by the smooth cells. However, the incorporation of smooth cells did not significantly reduce biofilm formation or the number of rugose cells (Fig. 7c). This finding is consistent with previous studies showing that *V. cholerae* prevented invasion of planktonic cells to the biofilm interior (Nadell *et al.*, 2015; Schluter *et al.*, 2015). Similarly, Irie *et al.* recently demonstrated that although *Pseudomonas aeruginosa* socially produce the PSL polysaccharide and its benefits are shared among producers, it is not cheatable (Irie *et al.*, 2016).

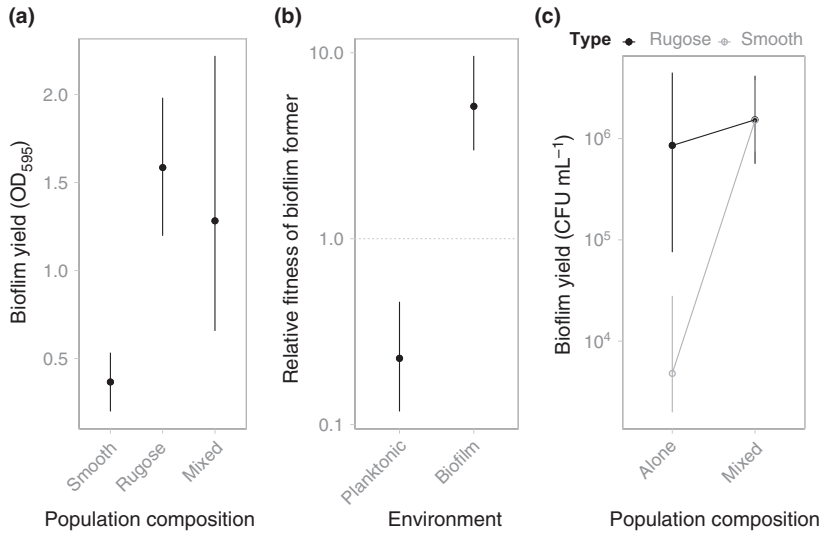

Fig. 7 Competition between evolved types. Biofilm-forming (rugose) and nonforming (smooth) types were isolated from evolved populations and grown both alone and together. (a) Populations containing the evolved biofilm-forming type produced significantly more robust biofilms than monocultures of the nonforming type. (b) When grown with the evolved nonforming type, the biofilm-forming type was disadvantaged in planktonic growth. However, the biofilm-forming type handily outcompeted the nonformer in colonizing a surface. (c) Although the nonforming type produced significantly weaker biofilms in monoculture, cultures initiated using an approx. 1 : 1 mixture of biofilm-forming and nonforming types produced biofilms that were as robust as those formed by biofilm-forming monocultures. Despite this enrichment for nonforming cells in mixed cultures, growth of the biofilm-forming type was not affected. In all plots, error bars indicate 95% confidence intervals.

Discussion

We have explored how resource abundance affects the evolution and maintenance of biofilm formation, a form of public goods cooperation. In both digital and microbial populations, cooperation was rare when a required resource was sparse (Figs. 2a and 5). However, cooperation persisted when resource was abundant. In both systems, the transition between these two evolutionary outcomes occurred rapidly at a critical level of resource, which suggests that selection for biofilm formation was different in these two environments. When simulated populations experienced a shift from resource-rich to resource-poor environments, cooperation rapidly decreased (Fig. 4). Conversely, when the environment became resource-rich, cooperation increased substantially.

Environmental conditions and other ecological factors drive selection for all traits, and social behaviours are no exception. In a clear demonstration of this relationship, Zhang and Rainey showed that the costly production of pyoverdine, an extracellular metabolite secreted by *Pseudomonas fluorescens*, is adaptive and cheatable in iron-limiting environments, but maladaptive when iron is more abundant (Zhang & Rainey, 2013). Similarly, several studies have shown that resource concentration can dictate where the interactions of cross-feeding microbial strains fall on the spectrum between mutualism and parasitism (Nielsen *et al.*, 2000; Bull & Harcombe, 2008; Hom & Murray, 2014). Notably, by adjusting amino acid concentrations, Hoek *et al.* recently altered the relationship between two yeast strains through several distinct outcomes from obligate mutualism to competitive exclusion (Hoek *et al.*, 2016).

The bimodal response that occurs in response to resource abundance suggests that below some critical level of resource, the benefits of biofilm formation do not compensate for the costs. In these environments, selection favours nonproduction (Figs. 2a and 5). Meanwhile, above critical resource levels, cooperation can be beneficial, which also presents opportunities for exploitation by cheaters. The plateau in cooperation that we observe in resource-rich environments may indicate that additional investment in biofilm production yields diminishing returns (Foster, 2004). Indeed, further production did not enhance survival once subpopulations had produced sufficient public goods to survive the environmental disturbance or to adhere to the pegs. A similar response to changes in resource concentration has been shown by cooperative traits that mediate plant infection by *Agrobacterium tumefaciens* (Platt *et al.*, 2012). In that study, harbouring a plasmid encoding those traits was costly when resources were limited, but beneficial in resource-rich environments.

Importantly, different cooperative behaviours or species can respond differently to changing environmental conditions. The proportion of *P. fluorescens* SBW25 wrinkly spreader morphotypes, which cooperatively

form a mat at the air–broth interface, increases linearly with resource concentration (Travisano & Rainey, 2000; Brockhurst *et al.*, 2008). When populations of these strains instead face different levels of environmental disturbance, cooperation shows a hump-shaped response, whereby cooperation peaks at intermediate disturbance (Brockhurst *et al.*, 2010).

Natural populations commonly reside in complex environments, where organisms are likely to experience ecological changes within their lifetime. Our work has provided further evidence that cooperative behaviours are occasionally maladaptive. In these instances, types can gain evolutionary stability by cooperating only when doing so is beneficial, and otherwise focusing on individual growth (Cornforth *et al.*, 2012; Darch *et al.*, 2012; Heilmann *et al.*, 2015). Indeed, several mechanisms have been identified that allow organisms to cooperate facultatively.

In *P. aeruginosa*, swarming is enabled by the cooperative secretion of biosurfactants. By producing these public goods only when carbon concentrations exceed what is necessary for growth, wild-type cells are immune to cheating, a phenomenon termed metabolic prudence (Xavier *et al.*, 2011). Whereas our Avida populations were not able to sense their environments, our evolved *Vibrio cholerae* types may be displaying a similar form of metabolic prudence. Our experiments indicate that the metabolic prudence may not just affect the expression of cooperative functions, but may extend to evolutionary timescales as well. Further experiments exploring how biofilm formers respond to fluctuating environments could highlight whether metabolic prudence leads populations down diverging paths based on environmental conditions.

Facultative cooperation can also be mediated by quorum sensing, a widespread form of signalling that allows microbial populations to respond to changes in population density and environmental conditions through the production and detection of small molecules (Waters & Bassler, 2005). Indeed, many cooperative traits are now understood to be regulated by quorum sensing systems (Bruger & Waters, 2015). Importantly, whether a cooperative trait is stabilized by quorum sensing in changing environments depends critically on the relationship between cooperative benefit and environmental change (Archetti & Scheuring, 2011; Cornforth *et al.*, 2012; Heilmann *et al.*, 2015). For instance, the formation of biofilms, which showed a sigmoidal response to changes in resource abundance in our experiments, is normally under quorum sensing control in *V. cholerae*. Using quorum sensing to selectively produce EPS only when doing so is beneficial, cooperator populations may gain evolutionary stability in fluctuating environments. Similarly, *P. aeruginosa* and *Vibrios* use quorum sensing to regulate the cooperative production of extracellular proteases, which exhibit a similar bimodal response to changes in carbon and population density (Darch *et al.*, 2012). Indeed, in *Vibrio harveyi*, quorum sensing control of extracellular proteases prevents defector invasion of the wild-type strain, whereas a constitutive cooperator is rapidly driven to extinction (Bruger & Waters, 2016). Conversely, quorum sensing does not regulate the production of iron-scavenging siderophores, which instead shows a linear response to resource abundance (Brockhurst *et al.*, 2008). Understanding how cooperation co-evolves with mechanisms like quorum sensing and metabolic prudence remains an important challenge that requires consideration of how selection for the cooperative behaviour in question is affected by the environment in which it occurs.

The relatedness of interacting individuals was an important factor in both of our systems. In the computational model, localized replication and a lack of mixing provided a degree of spatial structuring for all individuals. The formation of biofilms, however, allowed patches of producers to maintain persistent interactions, whereas patches of cheaters were continually fragmented by environmental disturbances. Similarly, the formation of biofilms created structuring within the populations of *V. cholerae*. As a result, producers were more likely to interact with closely related producers than with others. Kin were therefore more likely to benefit from cooperation, thus lessening the detrimental impact of cheaters. Further, populations can physically exclude cheaters and nonrelatives from the biofilm interior (Nadell *et al.*, 2015) and may induce biofilm formation in response to competition (Oliviera *et al.*, 2015). Several previous studies have demonstrated that spatial structuring and other mechanisms that support preferential cooperation among relatives can be crucial for maintaining cooperation (e.g. Kerr *et al.*, 2002; Nadell & Bassler, 2011; Strassmann *et al.*, 2011).

When biofilms enable populations to reach greater densities, cooperation may be further challenged by increased competition among kin (Taylor, 1992; Platt & Bever, 2009). Under these circumstances, cooperation can nevertheless be bolstered by pleiotropy (Dandekar *et al.*, 2012; Mitri & Foster, 2016), policing (Frank, 2003; Wang *et al.*, 2015) or nonsocial adaptation (Asfahl *et al.*, 2015; Hammarlund *et al.*, 2016). In our systems, the environmental disturbance created opportunities for the population to expand, allowing cooperators to escape competition from noncooperators (Video S1; Alizon & Taylor, 2008). Natural biofilms are often transient, and dispersal may provide a similar means of escape. To fully understand how cooperation evolves in biofilms, future studies therefore must consider all stages of the biofilm life cycle (see, e.g. Poltak & Cooper, 2011).

This work has demonstrated that in some environments, cooperation is simply a poor strategy. Determining how environmental change alters selection is crucial for gaining a broader understanding of how

cooperation evolves and is maintained in nature. This perspective is also necessary in the development of 'anti-infective' treatments that target cooperative virulence factors (Petersen *et al.*, 2009; Crespi *et al.*, 2014), as pathogens are likely to face a large diversity of ecological conditions within their host.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Figure S1 Snapshot of one population in our model during simulation. Through localized reproduction, patches of cooperators (dark grey) and cheaters (light grey) formed. Regions without sufficient levels of public good were decimated by a periodic environmental disturbance, creating empty space (white), which could then be colonized by neighboring individuals.
Figure S2 Cooperator proportion over time.
Figure S3 Rate of evolution in logic tasks.
Figure S4 Wild type growth in different LB concentrations.
Video S1 Supplementary material.

Data deposited at Zenodo: doi:10.5281/zenodo.236468

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