

Ecological drivers of the evolution of public-goods cooperation in bacteria

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Abstract. The role of ecological processes in the evolution of social traits is increasingly recognized. Here, we explore, using a general theoretical model and experiments with bacteria, the joint effects of disturbance frequency and resource supply on the evolution of cooperative biofilm formation. Our results demonstrate that cooperation tends to peak at intermediate frequencies of disturbance but that the peak shifts toward progressively higher frequencies of disturbance as resource supply increases. This appears to arise due to increased growth rates at higher levels of resource supply, which allows cooperators to more rapidly exceed the density threshold above which cooperation is beneficial following catastrophic disturbance. These findings demonstrate for the first time the importance of interactions between ecological processes in the evolution of public-goods cooperation and suggest that cooperation can be favored by selection across a wide range of ecological conditions.

Key words: *biofilms; collective action; experimental evolution; kin selection; selection experiment; social evolution.*

INTRODUCTION

Public-goods cooperation is widespread among microorganisms and explaining this is a major challenge for evolutionary biologists (Hamilton 1997, Maynard Smith and Szathmari 1997, Crespi 2001, West et al. 2006). The central problem is that investment in the public good incurs a metabolic cost for producing cells (cooperators), yet benefits all cells in the social group, whether or not they are producers. Thus, all else being equal, cells that make reduced (or zero) public-goods investments (cheats) are expected to have a fitness advantage, and hence invade populations of cooperators (Frank 1998). Kin selection provides a general solution to this social dilemma (Hamilton 1964): public-goods cooperation can be favored if the benefits of cooperation preferentially accrue to relatives with whom the cooperator shares genes. This is captured in Hamilton's rule which states that cooperation (or indeed any social trait) is favored when $r \times b > c$, where c is the personal fitness cost for the actor, b is the fitness benefit to the recipient, and r is the genetic relatedness between the actor and recipient. Thus, provided that the indirect benefit ($r \times b$) accruing from cooperation exceeds the direct cost (c) of investment, then public-goods cooperation can be favored by natural selection. Therefore, mechanisms that either lead cooperators to interact more often with other cooperators than with cheats (high relatedness), or cause an increase

in the benefits or a decrease in the costs of public-goods production, can favor its evolution.

It has recently been shown that ecological variables thought to affect the evolution of diversity, such as frequency of catastrophic disturbances (Connell 1978, Buckling et al. 2000) and resource supply (Tilman 1982, Hall and Colegrave 2006), can also influence the evolution of cooperation via their impacts upon relatedness and the costs and benefits of cooperation (Brockhurst et al. 2007, 2008). Cooperation was found to peak at intermediate disturbance frequencies: catastrophic disturbances caused population bottlenecks thereby increasing relatedness, however, if disturbances occurred too infrequently, de novo cheat mutants accumulated within populations; whereas, if disturbances were too frequent, population densities remained too low for the positive density-dependent benefits of cooperation to be realized (Brockhurst et al. 2007). Resource supply has been found to alter the cost of cooperation, which decreases with increasing resource supply, such that public-goods cooperation is more likely to evolve when resources are more abundant (Brockhurst et al. 2008).

However, ecological processes rarely act in isolation in nature, therefore it is crucial to understand their joint effects. In studies of diversity, theory predicts that the disturbance frequency that maximizes species richness can be influenced by resource supply, with peak species richness shifting to higher frequencies of disturbance as resource supply increases (Kondoh 2001, Worm et al. 2002). Such interactive effects of ecological variables help to explain unexpected patterns of species richness in nature (Proulx and Mazmuder 1998, Worm et al. 2002). As with diversity, it is not possible a priori to predict the

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joint effects of disturbance frequency and resource supply on the evolution of public-goods cooperation from their independent effects in isolation.

In this paper, we develop a general theoretical model of the joint effects of disturbance frequency and resource supply on the evolution of public-goods cooperation. We then test our predictions empirically using biofilm-forming genotypes of the bacterium *Pseudomonas fluorescens*. The *P. fluorescens* “wrinkly-spreader” (WS) morph forms a biofilm at the air–broth interface in heterogeneous microcosms (static glass vials containing growth medium; see Plate 1) through constitutive overproduction of cellulosic polymer (Rainey and Travisano 1998, Spiers and Rainey 2005). While overproduction of cellulosic polymer is individually costly (as demonstrated by the reduced exponential growth rate of WS relative to wild-type [Rainey and Rainey 2003, MacLean et al. 2004]), its production provides a group benefit to WS because colonization of the air–broth interface niche allows improved access to oxygen, a limiting resource (Rainey and Rainey 2003). Clonal WS biofilms have been found to be susceptible to rapid invasion by non-cellulose producing “smooth” (SM) genotypes that arise by mutation from WS over the course of several days (Rainey and Rainey 2003, Brockhurst et al. 2006). In this context, SM are cheats, gaining the benefit of inhabiting the air–broth interface while making no contribution to the integrity of the biofilm, which is significantly weaker in the presence of cheating SM genotypes (Rainey and Rainey 2003). The evolutionary emergence of WS cooperation is closely linked to resource supply, such that the proportion of de novo WS mutants observed in populations increases linearly with the nutrient concentration of growth medium (Travisano and Rainey 2000).

THEORETICAL MODEL AND ANALYSIS

Here, we develop a mathematical model of public-goods cooperation that incorporates the effects of catastrophic disturbance and resource supply. We are interested in providing an illustrative model that captures the essential features of the problem in a simple way, as this most readily permits clear and robust qualitative predictions that can be readily tested. In particular, we extend the basic model of Brockhurst et al. (2007), which examined the evolution of public-goods cooperation for a range of disturbance rates, to allow for variation in resource supply. For concreteness, our model is phrased in terms of microbial cooperation, but it is intended to have wider application. We assume a structured population of microbial cells in which each social group is founded by a single cell (or a small number of clonal cells), which then grows through clonal reproduction. We consider two kinds of cells: cooperators that produce public goods, and cheats that do not (see Plate 1). Both cell types have a baseline growth rate a (a monotonically increasing function of resource supply; setting $a = 1$ recovers exactly the model of

Brockhurst et al. 2007), but cooperators suffer a growth cost c due to public-goods contributions. If the absolute number of cooperators in a social group exceeds a threshold τ (defined as a multiple of initial group size), then all cells in the group enjoy a growth benefit b . We consider that loss-of-function mutations convert cooperators to cheats at a rate of μ per cell per standard time unit, and we assume that there is negligible mutation in the reverse direction. Finally, at time T the population is disturbed by a catastrophic event that destroys most cells and leaves a small number of survivors to found the next generation of social groups.

With these assumptions, we can write the growth rates of cooperators within a focal social group as

$$\frac{dx}{dt} = \begin{cases} (a - c - \mu)x_t & \text{if } x_t \leq \tau \\ (a + b - c - \mu)x_t & \text{if } x_t > \tau \end{cases} \quad (1)$$

and the growth rate of cheats within the social group is given by

$$\frac{dy}{dt} = \begin{cases} ay_t + \mu x_t & \text{if } x_t \leq \tau \\ (a + b)y_t + \mu x_t & \text{if } x_t > \tau \end{cases} \quad (2)$$

where x_t and y_t are the numbers of cooperators and cheats in the social group at time t , expressed as a multiple of initial group size (i.e., $x_0 + y_0 = 1$; Brockhurst et al. 2007).

We consider first a social group that is founded by a cooperator cell (or several cooperator cells). Here, the cooperators grow exponentially at per capita rate $a - c - \mu$ until reaching the threshold $x_t = \tau$; if this occurs at all, it happens at time $t^* = \ln(\tau)/(a - c - \mu)$. If catastrophe strikes before the threshold is crossed ($T \leq t^*$) then there is never any benefit for cooperation, and hence the proportion of cooperators is expected to decline to zero over successive growth periods (Brockhurst et al. 2007). Assuming that catastrophe strikes after the threshold is crossed ($T > t^*$), there is scope for cooperation to be favored. Here, the cooperators grow at per capita rate $a + b - c - \mu$, and hence at the moment of catastrophe the number of cooperators in the social group is $x_T^C = \tau \exp[(a + b - c - \mu)(T - t^*)]$ (the superscript C denotes a cooperator group). The cooperator-founded group generates a local population of cheats, which grows at total rate $ay_t + \mu x_t$ until time t^* , and hence reaches size $\mu\tau^{(c+\mu)/(a-c-\mu)} - 1)/(c + \mu)$ at this time. After the threshold is crossed at time t^* , the cheats grow at total rate $(a + b)y_t + \mu x_t$, and hence the number of cheats at the time of disturbance is

$$y_T^C = \left(\exp[(a + b - c - \mu)T] \times [\exp[(c + \mu)T] - 1] \mu \tau^{-b/(a-c-\mu)} \right) / (c + \mu).$$

We now consider a social group founded by a cheating cell (or a small number of cheats). The number of cheats in the group grows exponentially at a per capita rate a , hence there are $y_T^D = \exp(a \times T)$ cheats in the group at the moment of catastrophe (superscript D

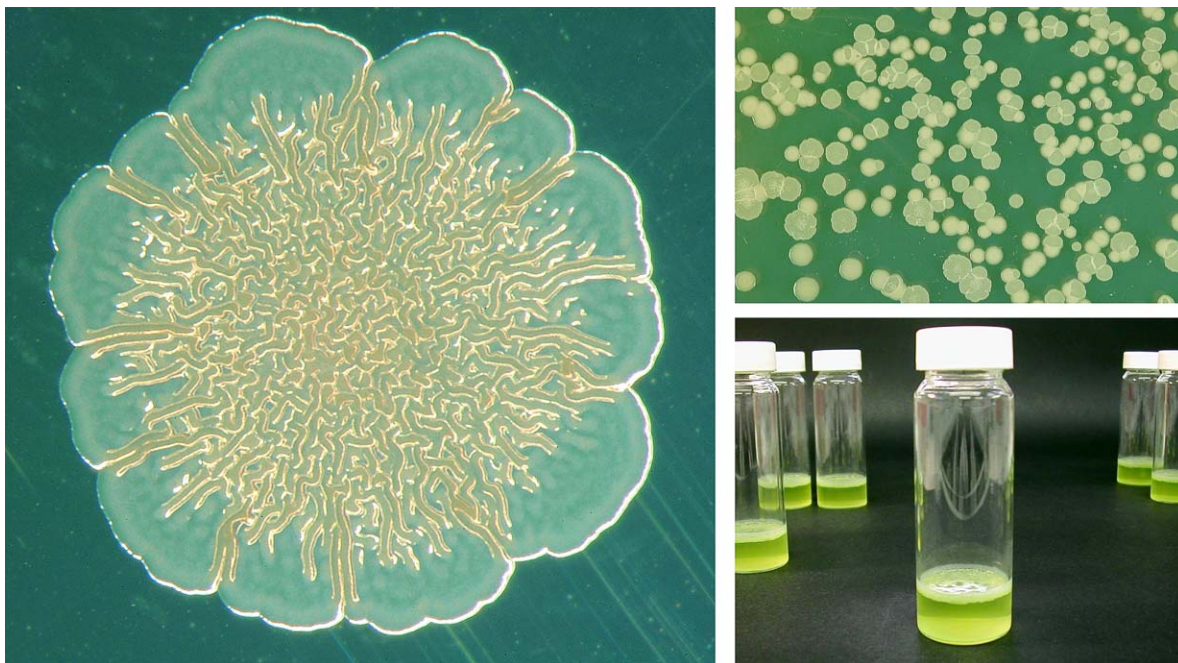


PLATE 1. (Left) A close-up of a wrinkly-spreader (cooperator) colony on an agar plate; (top right) wrinkly-spreader (cooperator) and smooth (cheat) colonies on an agar plate; (bottom right) a microcosm with a wrinkly-spreader biofilm growing at the air-broth interface. Photo credits: Andrew Spiers.

denotes a group founded by a cheat, or defector). Since no cooperators ever arise in this group, the number of cooperators at the moment of catastrophe is $x_T^D = 0$.

If the overall frequency of cooperators in the population at the beginning of the growth period is p , then the proportion of cooperator groups is p and the proportion of cheat groups is $1 - p$. The total proportion of cooperator cells at the moment of catastrophe after growth has occurred is then $p' = px_T^C / (px_T^C + y_T^C) + [1 - p]y_T^D$ and, because the catastrophic culling is random with respect to cooperation status, this is also the frequency of cooperators at the beginning of the next growth period. Hence, after successive rounds of growth and catastrophe, the equilibrium frequency of cooperators mediated by selection and mutation is given by solving $p = p' = p^*$, i.e.,

$$p^* = \frac{\left[(c + \mu) \left(e^{aT} - e^{(a+b-c-\mu)(T - \ln[\tau]^{a-c-\mu})} \right) \tau \right]}{\left[(c + \mu) \left(e^{aT} - e^{(a+b-c-\mu)(T - \ln[\tau]^{a-c-\mu})} \right) \tau - \left(e^{(a+b)T} - e^{(a+b-c-\mu)T} \right) \mu \tau^{-b/(a-c-\mu)} \right]} \quad (3)$$

We have already seen that cooperation is disfavored at all frequencies when $T < t^*$. Noting also that the right hand side of Eq. 3 tends to zero as $T \rightarrow \infty$, we recover the basic result of Brockhurst et al. (2007) that cooperation is disfavored at the extremes of low and high disturbance, and is most favored at intermediate disturbance frequencies. Further, the derivative of p^* with respect to a is

always positive, consistent with the finding by Brockhurst et al. (2008) that an increased resource supply, and hence a greater baseline growth rate, acts to increase the selective advantage of cooperation. Numerical analysis reveals an interaction between disturbance frequency and resource supply, such that the disturbance frequency that corresponds to the highest equilibrium frequency of cooperators is an increasing function of resource supply; in other words, when resources are abundant, increasing disturbance is predicted to lead to increased cooperation over most of the range of disturbance frequencies. The separate disturbance, resource supply and disturbance \times resource supply effects are illustrated by the numerical example in Fig. 1a.

EXPERIMENTAL MATERIALS AND METHODS

Disturbance frequency by resource supply factorial selection experiment.—Four independent WS genotypes were isolated as previously described (Brockhurst et al. 2007). Experimental populations were initiated with 1×10^6 cells of one of the isolated WS genotypes grown for 18 h at 28°C under shaken conditions in a KB microcosm (30-mL glass universal containing 6 mL of King's B nutrient media containing 10 g/L glycerol, 20 g/L proteose peptone, 1.5 g/L K_2HPO_4 , and 1.5 g/L $MgSO_4$). Experimental populations were then propagated under one of the following disturbance regimes. After thorough homogenization, 6 μ L of culture was transferred to a fresh microcosm every 1, 2, 4, 8 days, or not at all during a 16-day period at each of the following

resource supply regimes: 0.125 \times , 0.25 \times , 0.5 \times , 1 \times , and 2 \times standard KB, generated by serial dilution of KB medium into M-9 salt solution. After 16 days, populations were homogenized and plated onto KB agar and the frequencies of WS and SM colonies counted.

Testing the density threshold assumption.—Our model assumes that the benefits of cooperation are positive density dependent and can only be realized once the cooperator population overcomes a density threshold. To explicitly test this we compared short-term growth rates of WS in static and shaken KB microcosms at high ($\sim 1 \times 10^6$ cells per microcosm) and low ($\sim 1 \times 10^5$ cells per microcosm) starting densities. Six populations were founded with 1×10^6 WS cells and six populations were founded with 1×10^5 WS cells that had been previously grown for 18 h at 28°C under shaken conditions in a KB microcosm. Half of the microcosms at each starting density were incubated statically while the other half were shaken for 8 hours at 28°C. Starting (i.e., 0 hours) and final (i.e., 8 hours) population densities were estimated by plating dilutions onto KB agar. Malthusian parameters (m) were calculated as $m = \ln(\text{final density}/\text{starting density})$.

Growth in depleted medium.—Because a previous study identified that cell death occurred at low disturbance at intermediate resource supply (Brockhurst et al. 2007), we compared growth of WS and SM genotypes in depleted media to assess whether this contributed to patterns observed in this study. Depleted medium was prepared by statically incubating each of the four WS in replicate KB microcosms for 12 days, after which time populations were thoroughly vortexed and the media filter-sterilized using 0.2- μm filter syringes. Two milliliters of depleted medium each was pipetted into eight sterile 30-mL glass universals. Four populations were initiated with 1×10^7 cells of one of the four WS genotypes and four populations were initiated with 1×10^7 cells of the ancestral SM grown for 18 h under shaken conditions. Prior to incubation, each population was plated onto agar to estimate starting cell density. After 24 hours of static incubation at 28°C, populations were plated onto agar to estimate final cell densities. Malthusian parameters (m) were calculated as $m = \ln(\text{final density}/\text{starting density})$.

RESULTS

Disturbance frequency and resource supply had significant interactive effects on the evolutionary maintenance of cooperation (Fig. 1b, Table 1). The predicted hump-shaped relationship between disturbance frequency and cooperation was maintained across much of the resource supply gradient (Table 1; quadratic effect of disturbance frequency, $P < 0.001$), however, this relationship was most evident at intermediate and high levels of resource supply (0.5 \times to 2 \times standard concentration) and was weakened at low levels of resource supply (0.125 \times and 0.25 \times standard concentration; Fig. 1b, Table 1; quadratic disturbance frequency \times linear

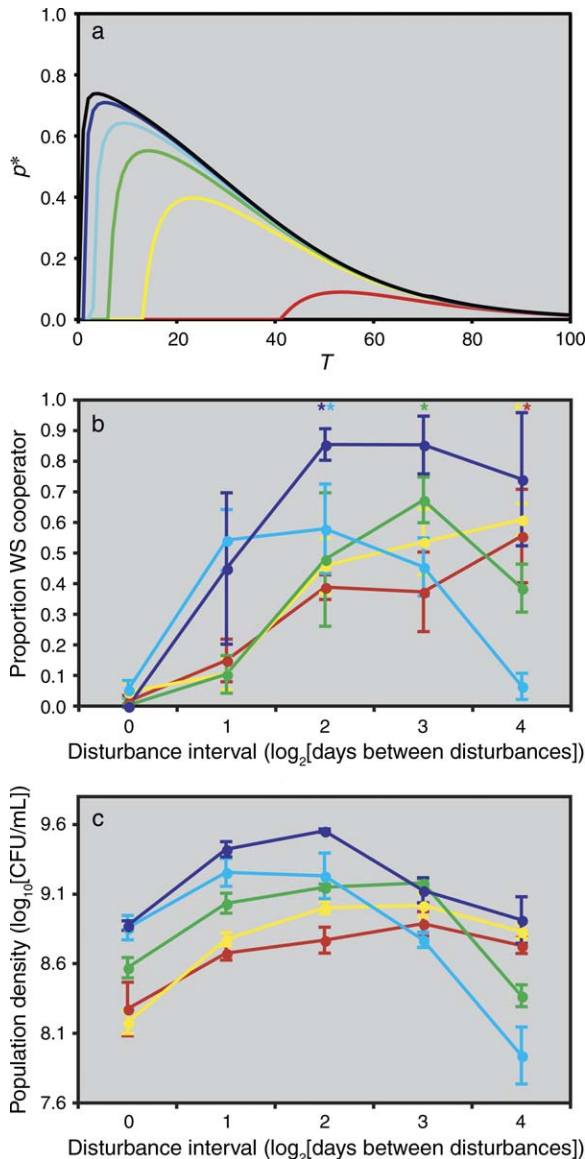


FIG. 1. Interactive effects of resource supply and disturbance frequency. (a) Theoretical predictions: the equilibrium level of cooperation (p^*) is a dome-shaped function of disturbance interval (T) and increases with greater resource supply (reflected by a greater baseline growth rate, a). Peak cooperation levels occur at a disturbance frequency ($1/T$) that is increasing with greater resource supply (higher a), i.e., over a greater range of disturbance frequencies there is a positive correlation between disturbance frequency and cooperation. Colored lines represent values of a : 0.2 (red), 0.5 (yellow), 1 (green), 2 (light blue), 5 (dark blue), and 10 (black). Other parameters were set at $b = 0.1$, $c = 0.05$, $\tau = 10$, and $\mu = 0.01$. (b) Experimental results: cooperation. Dots represent proportions of *Pseudomonas fluorescens* “wrinkly-spreader” morph (WS; mean \pm SE) at 0.125 \times (red), 0.25 \times (yellow), 0.5 \times (green), 1 \times (light blue), and 2 \times (dark blue) standard resource concentrations. Colored asterisks denote the highest mean cooperators at each resource supply. (c) Experimental results: population density. Dots represent \log_{10} population densities (mean \pm SE) (colors are as in panel b).

TABLE 1. General linear model of arcsine-square-root-transformed proportion of *Pseudomonas fluorescens* “wrinkly-spreader” (WS) morph, fitting founding genotype (G) as a random factor and \log_2 (disturbance frequency) (D) and \log_2 (resource supply) (R) as covariates, using sequential sums of squares for tests.

Factor	df	MS	F	P
G	3	0.09	2.61	0.056
D	1	4.27	122.44	<0.001
R	1	0.47	13.33	<0.001
D × D	1	2.18	62.45	<0.001
R × R	1	0.19	5.51	0.021
D × R	1	0.01	0.26	0.609
R × D × D	1	0.53	15.16	<0.001
D × R × R	1	0.18	5.19	0.025
D × D × R × R	1	0.00	0.02	0.884
Error	88	0.03		

resource supply interaction, $P < 0.001$). In accordance with our theoretical model, peak levels of cooperation, determined as the highest mean cooperator proportion, were observed at progressively higher frequencies of disturbance as resource supply increased (Fig. 1b; Pearson’s correlation of disturbance frequency supporting highest mean cooperator proportion and resource supply; $r = 0.938$, $P = 0.02$).

Our model assumes the existence of a density threshold below which the benefits of cooperation cannot be realized. We explicitly tested this by comparing short-term growth rates of WS at high and low starting densities in static and shaken microcosms. In line with the benefits of cooperation being positive density dependent, we observed higher growth rates of WS when grown from high compared to low starting density in static microcosms but not in shaken microcosms where biofilm formation was prevented (Fig. 2; two-way ANOVA, starting density × microcosm type interaction, $F_{1,8} = 10.166$, $P = 0.01$). Also consistent with the existence of a threshold density, in our evolved lines, cooperation was always disfavored under very high frequency disturbance (Fig. 1b) and the proportion of cooperators was positively correlated with population density (Figs. 1b, c; Pearson’s correlation of means; $r = 0.647$, $P < 0.001$). This suggests that higher growth rates due to increased resource supply allowed sufficient densities to be reached at shorter disturbance intervals. The cost of cooperation was also expected to decrease with increasing resource supply (Brockhurst et al. 2008), and in support of this, the frequency of cooperation generally increased with increasing resource supply (Fig. 1b, Table 1; linear effect of resource supply, $P < 0.001$).

At intermediate levels of resource supply, most notably, 1× standard concentration, we observed sharp declines in density at low frequency disturbance (Fig. 1c). Such density declines were associated with low cooperator frequency causing an unexpected U-shaped relationship between resource supply and proportion of cooperators under 16-day disturbance intervals (Fig. 1b, Table 1; quadratic effect of resource supply, $P = 0.021$,

quadratic resource supply by linear disturbance frequency interaction $P = 0.025$). This suggested that WS suffered higher rates of cell death compared to SM. This possibly represented a further pleiotropic cost of the WS cooperative phenotype beyond the obvious metabolic demands of cooperation considered in our theoretical model. We tested whether cooperators and cheats had different susceptibilities to low disturbance conditions by culturing WS and SM separately in 12-day-old used KB media that had been filter sterilized. While SM had a positive growth rate in this depleted media, WS cells died and declined in density over 24 hours (SM Malthusian parameter = 1.03 ± 0.24 [mean ± SE]; WS Malthusian parameter = -0.50 ± 0.22 ; two-sample t test, $t = 4.71$, $df = 6$, $P = 0.003$). This suggests that greater turnover of SM relative to WS in stationary phase under low disturbance conditions likely contributed to the invasion of cheats at intermediate resource-supply levels.

DISCUSSION

It is increasingly recognized that variation in ecological parameters are likely to have significant effects on the evolution of social traits. Our results suggest that disturbance frequency and resource supply have strong interactive effects on the evolution of cooperation that cannot be extrapolated from their independent effects in isolation. Cooperation increases with increasing resource supply, and tends to peak at intermediate frequencies of disturbance, but crucially, cooperation is favored at progressively higher frequencies of disturbance as resource supply is increased. At very low levels of resource supply we observed a positive relationship between cooperation and disturbance frequency, however, it seems likely that this represented just one side of a unimodal relationship, and that at even lower frequencies of disturbance, cooperation would

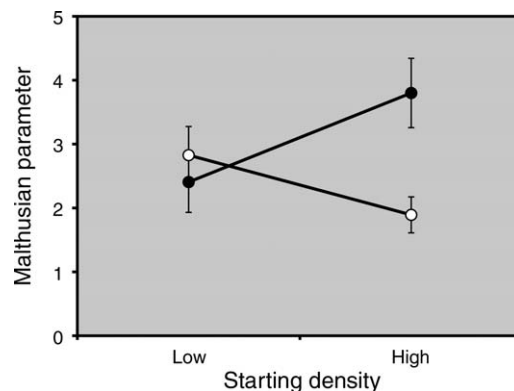


FIG. 2. Effects of starting density on short-term growth rates of WS in static and shaken microcosms. Dots represent mean Malthusian parameters of WS (mean ± SE) after 8 hours of growth from high (1×10^6 WS cells per microcosm) and low (1×10^5 WS cells per microcosm) starting densities in shaken (open symbols) and static (filled symbols) microcosms. Malthusian parameters (m) were calculated as $m = \ln(\text{final density}/\text{starting density})$.

have declined as predicted by our theoretical model. Why should cooperation peak at progressively higher frequencies of disturbance with increasing resource supply? Our theoretical model suggests that this pattern is due to increases in growth rate with increasing resource supply, such that less time is required between catastrophic disturbance events for cooperators to exceed the minimum density threshold for successful cooperation. Our findings may therefore be limited to systems where there exists a positive relationship between growth rate and resource supply, unlike, for example, chemostats.

Consistent with the existence of a minimum density threshold for successful cooperation, cooperation was always disfavored at very high frequency disturbance, and a positive correlation was observed between population density and cooperation. We also observed positive density dependent short-term growth rates of WS when grown in static microcosms, but not in shaken microcosms where biofilm cooperation was prevented. Such positive density dependent benefits of cooperation have been observed in a range of other microbial public-goods traits, including other bacterial biofilms (Cui et al. 2001, Li et al. 2001), and *Myxococcus xanthus* fruiting bodies (Kadam and Velicer 2006). However, in certain cases, the fitness benefits of public-goods cooperation may not be subject to a positively density dependent threshold. A possible example is invertase secretion in yeast, which breaks down sucrose into glucose and fructose. Here, public-goods production can be favored at low density because it yields a direct fitness benefit to producers (Greig and Travisano 2004), provided there is relatively limited diffusion of the public-good away into the environment (notably this experiment was carried out on agar plates rather than in liquid media). In such cases, it is possible that cooperator fitness would be highest under very high frequencies of disturbance where direct benefits can be accrued and high relatedness maintained. However, it is likely that the cost of public-good production will decrease with increasing resource supply; thus the positive relationship between resource supply and cooperation is still expected (Brockhurst et al. 2008).

Because our theoretical model is intended to be relatively general, it inevitably ignores certain specific details of our experimental system. Most notably, at intermediate levels of resource supply, we observed high death rates of WS cells compared to SM cells under low disturbance conditions. Toxic by-products of metabolism are likely to accumulate in low disturbance environments, however, it is unclear, given that the WS phenotype is typically caused by a single mutation (Bantinaki et al. 2007), how these toxins differentially cause cell death in WS and SM. Perhaps more likely, is that both WS and SM are subject to a certain level of toxin mediated cell death under low disturbance, but that SM are better able to scavenge and metabolize available nutrients and maintain some population turnover. This is consistent with a known pleiotropic

effect of the WS phenotype, namely, reduced breadth of catabolic functions, which are fully restored upon reversion to SM (MacLean et al. 2004). That the theoretical model recovers the basic qualitative results of the experimental study, despite neglecting such details, suggests that the described effects of disturbance frequency, resource supply, and their interaction, are robust and not system specific.

It is interesting to compare our results with those of studies of species diversity, where the joint effects of disturbance frequency and resource supply have been considered extensively. Of particular note is that both theoretical models (Kondoh 2001, Worm et al. 2002) and empirical data (Worm et al. 2002) predict that peak species richness should occur at progressively higher frequencies of disturbance as resource supply increases, which is similar to the pattern observed here for cooperation. These species diversity models assume a trade-off between colonization ability and competitiveness, such that good colonizers are favored under high disturbance frequencies and good competitors are favored under low disturbance frequencies. Increasing resource supply increases the colonization rate of poor colonizers (or good competitors) allowing these species to better persist within the community at higher frequencies of disturbance. In our study, cooperators and cheats cannot be considered directly equivalent to either good colonizers or good competitors, for example cheats are favored at both high and low disturbance. Nevertheless, in line with the species diversity models we find that increased resource supply effectively boosts the colonization ability of cooperators by increasing the rate at which the threshold density for successful cooperation is attained following catastrophic disturbance. It is important to note however, that our findings apply only to catastrophic disturbances causing random mass mortality, rather than disturbances causing selective mortality (Connell 1978).

An important implication of our results is that for a wide range of disturbance frequencies and levels of resource supply, public-goods cooperation can be favored by natural selection. Both ecological parameters are known to vary widely in nature. Therefore, across a heterogeneous landscape, our findings suggest that at any given time there are likely to exist spatial refuges in which cooperation can be maintained. These refuges may provide foci from which cooperation can subsequently spread to other regions of the landscape. We suggest that considering the evolution of social traits at a landscape-scale is a crucial next step in understanding their widespread maintenance in natural populations, and presents novel challenges to be explored in future studies.

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