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DENSITY DEPENDENCE AND COOPERATION: THEORY AND A TEST WITH BACTERIA

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Although cooperative systems can persist in nature despite the potential for exploitation by noncooperators, it is often observed that small changes in population demography can tip the balance of selective forces for or against cooperation. Here we consider the role of population density in the context of microbial cooperation. First, we account for conflicting results from recent studies by demonstrating theoretically that: (1) for public goods cooperation, higher densities are relatively unfavorable for cooperation; (2) in contrast, for self-restraint–type cooperation, higher densities can be either favorable or unfavorable for cooperation, depending on the details of the system. We then test our predictions concerning public goods cooperation using strains of the pathogenic bacterium *Pseudomonas aeruginosa* that produce variable levels of a public good—iron-scavenging siderophore molecules. As predicted, we found that the relative fitness of cheats (under-producers) was greatest at higher population densities. Furthermore, as assumed by theory, we show that this occurs because cheats are better able to exploit the cooperative siderophore production of other cells when they are physically closer to them.

KEY WORDS: **Kin selection, population structure, *Pseudomonas aeruginosa*, public goods, siderophores.**

Explaining the evolution and maintenance of cooperation is one of the most important and long-standing problems in evolutionary biology (Maynard Smith and Szathmàry 1995; Hamilton 1996). Although a profusion of theoretical work in the past five decades has led to a good theoretical overview of the different mechanisms by which cooperation can arise and persist, much of this theory remains to be tested (Sachs et al. 2004; Lehmann and Keller 2006; West et al. 2007b). One area that has attracted a lot of theoretical attention is how selection for cooperation can be affected by the ecology and demography of populations (see reviews by Frank 1998; Rousset 2004; and Lehmann and Keller 2006). Genetic composition, density, substructuring, and the degree of mixing between individuals can all affect the spread and stability of behavioral strategies within a population; but it can be difficult to predict, from such general theory, precisely how such effects would apply in the context of particular cooperative systems (Leimar and Hammerstein 2006; West et al. 2007b). Moreover, it has been difficult to test these ideas empirically, because of the inherent complexities of experimentally manipulating population structure in traditional model systems such as vertebrates or insects.

In this article, we focus on the role of population density in the evolution of cooperation in microbes. It has recently been realized that microbes exhibit a striking range of cooperative behaviors,

which offer excellent opportunities for testing social evolution theory (Crespi 2001; West et al. 2006). This is because mutants that cooperate less (cheats) can be isolated from natural populations or artificially generated, the costs and benefits of cooperation can be easily manipulated experimentally, and the resultant fitness consequences can be monitored over many generations (Velicer 2003; West et al. 2006; Foster et al. 2007). Microbes are particularly useful for investigating the role of population demography in the evolution of cooperation, and recent results have provided clear support for the importance of factors such as population structure (Griffin et al. 2004; Brockhurst et al. 2007; Diggle et al. 2007b; Gilbert et al. 2007; Kümmerli et al. 2009a), assortment within populations (Strassmann et al. 2000; Queller et al. 2003; Mehdiabadi et al. 2006), and the relative frequency of cooperators versus cheats in a population (Velicer et al. 2000; Dugatkin et al. 2003, 2005; MacLean and Gudelj 2006; Diggle et al. 2007b; Ross-Gillespie et al. 2007; Kümmerli et al. 2009b; Rumbaugh et al. 2009). With population density, however, the picture is less clear. Although one recent study in yeast reported cheats having a higher relative fitness at higher population density (positive density dependence; Greig and Travisano 2004), another yeast study found that cheats had a higher relative fitness at lower population densities (negative density dependence; MacLean and Gudelj 2006; MacLean 2008).

Our first objective in this article is to examine theoretically when positive or negative density dependence should be observed. Our overall aim is to produce illustrative models that can explain why differing patterns of density dependence have previously been reported, and to clarify when clear a priori predictions can be made for future empirical studies. We develop mathematical models, rather than just verbal arguments, because we wish to make explicit the multiple selective factors at work. We consider two different "social dilemma" scenarios that are likely to be important in microbes. In the first scenario, individuals cooperate by manufacturing products that can be used by their neighbors (i.e., public goods). Numerous extracellular factors produced and released by bacteria constitute public goods (West et al. 2007a). The second scenario involves a preexisting, finite, public resource. Here, individuals can cooperate by showing "self-restraint" and using the resource more efficiently, as opposed to quickly but relatively inefficiently (the "tragedy of the commons" scenario; Hardin 1968; Rankin et al. 2007). An example of this scenario occurs where organisms can switch between alternative metabolic pathways of respiration or fermentation (West et al. 2007a; MacLean 2008).

In the second part of the article, we test a major prediction arising from our model for the public goods scenario. Specifically, we test the prediction that the relative fitness of cheats, that under-produce an essential public good, is positively dependent on population density. We use as our model cooperative trait the production of siderophores in the bacterial pathogen *Pseudomonas aeruginosa* (West and Buckling 2003; Griffin et al. 2004). Siderophores scavenge iron from the environment, most of which is otherwise unavailable to bacteria because it is either actively withheld by hosts or is in the insoluble Fe(III) form (Guerinot 1994; Ratledge and Dover 2000). Siderophores are secreted extracellularly, diffuse freely, and can be taken up by any neighboring cell—although their diffusion is limited in viscous environments (e.g., ~6 mm over 24 h in 1% agar; R. Kümmerli et al., unpubl. data). Previous work has shown that siderophore production is a cooperative behavior, vulnerable to exploitation by cheats (Griffin et al. 2004; West et al. 2007c; Ross-Gillespie et al. 2007; Kümmerli et al. 2009a,b). As well as testing for positive density dependence, we also test the underlying assumption of our theoretical model: that cheat fitness is contingent on the physical proximity to cooperators and the siderophores that they produce. Although our theoretical work examines both public goods and self-restraint, we test only the public goods model, because that is where the clearer a priori prediction can be made (see below), and because this is part of our longer term project on the evolution of siderophore production.

Models and Analyses

In this section, we develop models that can explain why differing patterns of density dependence have previously been reported, and clarify when clear a priori predictions can be made for future empirical studies. Our aim is to develop illustrative models that emphasize the key elements of the underlying biology, and make clear why certain ecological conditions lead to density dependence (via their effect on relatedness and competition), rather than just assuming that the cost or benefit of behavior varies with density (Brown and Johnstone 2001; Brockhurst et al. 2007). The advantage of such illustrative models is that they avoid the potential semantic confusion that can arise with purely verbal arguments, but are still general enough to allow us to make generalizations across systems (i.e., our aim is not to capture all the finer details of a particular system). We first give a general equation for the fitness of cheats and then consider the specific cases of public goods production and self-restraint over resource use.

RELATIVE FITNESS OF CHEATS

Let the personal fitness w of an individual cell be a function of its cooperation strategy X and the average cooperation strategy Y of its social group, that is $w = W(X,Y)$. We define a "cooperator" as investing more, and a "cheat" as investing less, into cooperation (see West et al. 2007c for a discussion of terminology). In particular, we assume that cheats invest an amount x into cooperation whereas cooperators invest $x + \delta x$, where $\delta x > 0$, and we denote the average strategy of the cheat's social group as y and

the cooperator's social group as $y + \delta y$. Thus, the relative fitness of cheats can be expressed as

$$v(x, y, \delta x, \delta y) = \frac{W(x, y)}{W(x + \delta x, y + \delta y)} = 1 - \left(\frac{\partial W}{\partial X}\delta x + \frac{\partial W}{\partial Y}\delta y\right) / W(x, y) + O\left(\delta^2\right), \quad (1)$$

where the partial derivatives are evaluated at $X = x$ and $Y = y$. The partial derivative $\partial W/\partial X$ describes the personal fitness impact of increasing one's own level of cooperation (X), holding fixed the cooperation level of one's social partners (Y). Conversely, $\partial W/\partial Y$ describes the personal fitness impact of increasing the level of cooperation by one's neighbors (Y), while holding fixed one's own cooperation level (X). Noting that $\delta y/\delta x = dY/dX + O(\delta)$, and denoting $dY/dX = R$, that is the "whole-group" relatedness,(see below) relative fitness can be expressed in the form

$$v(x, y, \delta x, \delta y) = 1 - \left(\frac{\partial W}{\partial X} + \frac{\partial W}{\partial Y}R\right)\delta x / W(x, y) + O\left(\delta^2\right). \quad (2)$$

Whole-group relatedness measures an individual's average relatedness to its group—including itself (formally, the expected relatedness of two cells randomly drawn from the social group, with replacement; Taylor and Frank 1996; Pepper 2000; Rousset 2004), and hence is a function of both relatedness and number of social partners. In particular, we can write $R = 1/n + ((n - 1)/n)r$, where n is the total number of individuals in the social group and r is the "others-only" relatedness (the relatedness of an individual to the group, excluding itself—formally, the expected relatedness of two cells randomly drawn from the same group, without replacement; Pepper 2000).

PUBLIC GOODS

We consider the scenario in which individuals cooperate by manufacturing products that can be used by their neighbors (i.e., public goods; Dionisio and Gordo 2006), as occurs with the numerous extracellular factors produced by bacteria (West et al. 2007a). First, we assume that the production of public goods by an individual or group is equal to the value of its cooperation strategy, i.e., X or Y. Second, we assume that the total amount nY of public goods produced by the group is shared equally among its n members, so that each cell receives an amount Y of public goods irrespective of its own investment. Third, we assume that the growth benefit of public goods is a power function of the quantity taken up by the cell, with exponent a and scaled by an amount b, and added to a baseline growth equal to $1 - b$. The exponent a describes how sustained are the fitness returns of increased access to public goods (with lower a indicating greater diminishing returns; empirical results suggest $a < 1$, Ross-Gillespie et al. 2007) and the scaling parameter b describes the relative growth benefit of public goods. Finally, we assume that the production of an amount of public goods X incurs a personal multiplicative growth cost cX. Putting all this together yields the fitness function

$$W(X, Y) = (1 - b + bY^a)(1 - cX). \quad (3)$$

In the Appendix, we show that the relative fitness of cheats increases with cell density (positive density dependence). This is because, with increasing cell density, the public good availability within a social group is less dependent upon any one cell and is more dependent upon the average investment made by the other cells in its social group, hence giving cheats more opportunities to exploit cooperative social partners.

More generally, we find that the positive density-dependence effect is decreasing in magnitude as the population becomes more genetically structured (higher r). This is because cheats will be more likely to be in a social group with other cheats, hence reducing the opportunities for cheats to exploit cooperators (more formally, the group average investment will be closer to the strategy of the focal cell). Greig and Travisano (2004) argued that density alters the relative fitness of cheats because it alters "sociality"—our model makes explicit that the driving factor is the extent to which cheats can exploit cooperators, and that although this increases with density, the extent to which it does so can be reduced by population structuring.

These results are based upon the sufficient (but not necessary) assumption that others-only relatedness: (1) is not unity ($r < 1$); and (2) does not increase with increasing cell density ($dr/dn \leq 0$). Were we to relax (2) and allow others-only relatedness to increase with cell density, it is conceivable that this could occur at a rate sufficient to counterbalance the dilution of a single cell within the social group, and hence give rise to relative cheat fitness that is negatively density dependent. In the Appendix, we also consider the possibility that, at low cell density, not all public good molecules will be used by cells. This recovers the same qualitative predictions, provided that public goods are sufficiently beneficial in their action. Finally, we emphasize that we have assumed that various model parameters (a, b, and c) are density independent, whereas a more realistic model might relax this assumption. Our rationale for this is that we do not wish to force density dependence into the model, but rather have it emerge naturally as a result.

SELF-RESTRAINT

We now consider a tragedy of the commons scenario, where cooperation is in the form of self-restraint in resource use (Frank 1996, 1998), as occurs with the acquisition of energy from the degradation of organic substrates (Pfeiffer and Bonhoeffer 2003; MacLean and Gudelj 2006; MacLean 2008). In this case, the problem is that although respirers (cooperators) obtain a higher yield of ATP, respiro-fermenters (cheats) are able to use resources more quickly, and hence gain a greater share of the resources, but with a much smaller ATP yield. This trade-off between efficient

or fast use of resources will occur in all heterotrophic organisms, and has recently been examined from a social perspective in yeast (MacLean and Gudelj 2006; MacLean 2008).

First, we assume that cell density determines the relative (per cell) availability of resources, with resources being relatively abundant when the cell density is low, and relatively scarce when the cell density is high. We capture this by assuming that a proportion $1 - \exp(-kn(1 - Y))$ of the social group's resources is used up, where $1 - Y$ is the group average effort allocated into resource acquisition (and hence Y represents the degree of "self-restraint," or prudence, in resource use), and k is a positive constant describing how rapidly the resources become limiting as cell density increases (larger k represents greater exhaustion of resources). Second, we assume that each cell acquires a fraction of the group's total resources in proportion to its relative resource allocation effort, $1 - X$. Finally, we assume that the conversion of resources into growth is more efficient if the cell allocates relatively less into resource acquisition (i.e., a trade-off between rate and yield; Pfeiffer et al. 2001; MacLean and Gudelj 2006; MacLean 2008). In particular, we write the conversion efficiency benefit as a power function of acquisition effort, with exponent a and scale parameter b, and express this relative to a baseline conversion efficiency of $1 - b$. Putting this together gives the fitness function

$$W(X, Y) = \frac{1 - X}{n(1 - Y)}(1 - \exp(-kn(1 - Y)))(1 - b + bX^a). \quad (4)$$

In the Appendix, we show that density has two contrasting effects on the relative fitness of cheats—individuals that take more than their fair share of group resources. First, increasing cell density leads to a decrease in resource availability to each cell, which favors cooperators that use resources in a more prudent way. Second, increasing cell density leads to larger social groups (lower whole-group relatedness, $dR/dn < 0$), which favors cheats, as also occurred in the public goods model. As in the public goods model, this second effect is based upon the (sufficient, but not necessary) assumption that the relatedness to other cells in the group does not increase with cell density ($dr/dn \leq 0$). Consequently, because these two effects act in opposite directions, no clear a priori prediction can be made over whether the relative fitness of cheats should be positively or negatively density dependent. This contrast with the previous suggestion that restraint over resource use should lead to the fitness of cheats decreasing with density (negative density dependence; MacLean and Gudelj 2006).

Our above discussion has assumed that the relatedness to other cells in a group does not increase with cell density ($dr/dn \leq 0$). However, it is possible that this could occur, if situations that led to higher densities also led to more structured populations. If the relatedness to other cells increased with cell density at a sufficient rate, then this would offset the dilution of the focal cell within its social group ($1/n$), so that the whole-group relatedness increases (or stays the same) as cell density increases. Specifically, because whole-group relatedness (R) is related to others-only relatedness (r) by $R = 1/n + ((n - 1)/n)r$, then $dR/dn \geq 0$ requires that $dr/dn \geq (1 - r)/(n(n - 1))$. In that case, we could make the clear a priori prediction that increasing cell density leads to a decreasing relative fitness of cheats. Overall, however, no firm prediction can be made for the sign of density dependence without specifying the precise relationship between cell density and relatedness.

MacLean and Gudelj (2006) have previously argued that, in yeast, the fitness of cheats is lower at higher densities (negative density dependence) because the metabolic pathway employed by cheats yields toxic byproducts that decrease fitness as they accumulate, and this accumulation of toxins is increased at higher population densities. Our results clarify that toxic byproducts are not required to produce density dependence; rather, this pattern can arise for the reasons discussed above. However, toxic byproducts introduce a third factor that influences density dependence, and will increase the region of parameter space over which negative density dependence is predicted.

Methods

In the theoretical section we showed that a clear a priori prediction could be made for the case of public goods production, particularly in the case of "necessary" public goods (where $b \to 1$). Specifically, if relatedness between potentially interacting cells is held constant, then the fitness of cheats should increase with higher population densities. In this section, we test this prediction, by examining how the relative fitness of cheats varies depending upon density, while holding the initial starting proportion of cheats constant. In addition, we test the underlying assumption of our theoretical model, that cheat fitness is increased when they are growing closer to cooperators and the siderophores that they produce (i.e., that the effect of density is through its influence on proximity—higher density leads to cells being more likely to interact and therefore reduces whole group relatedness).

DESCRIPTION OF STRAINS

We replicated all our experiments with three independently derived cooperator–cheat strain pairs to demonstrate that observed effects are not strain-specific (for further strain details, see Ross-Gillespie et al. 2007). Briefly, these pairs were derived from the well-categorized *P. aeruginosa* isolates PA01 ATCC15692 (pairs "A" and "B") and UCBPP-PA14 (pair "C"). Although cooperators ("+") retained the siderophore production proficiency of their wild-type progenitors, cheat strains ("−") had mutations conferring defective production of the primary siderophore, pyoverdine, induced, respectively, by means of ultraviolet and transposon

mutagenesis (pair A; Rella et al. 1985; Hohnadel et al. 1986), deletion of the pyoverdine synthesis gene pvdD (pair B; Ghysels et al. 2004), or laboratory selection (pair C; Ross-Gillespie et al. 2007). In each case, cheat strains formed white colonies when grown on Kings B agar, making it possible to distinguish them from cooperator colonies, which appear green.

EXPERIMENTAL PROTOCOLS

Our first experiment tested our model's prediction that cheat fitness would be positively correlated with overall cell density in spatially structured populations (positive density dependence). For each strain pair, we cultured + and − from freezer stock in 6 mL standard Kings B solution in 30 mL glass universal vials, incubating overnight in an orbital shaker (37°C, 200 rpm). After 24 h, the cultures were vortexed and combined to obtain a master mix culture of – and + at a volumetric ratio of 1 in 10 (the relatedness between cells, r, is therefore > 0 and is held approximately constant with starting conditions across treatments). We assessed, posthoc, the actual proportion of – cells in this master mix by taking multiple samples, growing these overnight on Kings B agar plates, and then counting colony-forming units (CFUs), which could be categorized as either + or – on the basis of color and morphology (95% CI for realized proportions: pair A: 0.1266–0.1636; pair B: 0.0397–0.0670 pair C: 0.0572–0.0932)

To create conditions in which the cooperative trait (siderophore production) was important to fitness, we prepared culture media in which iron was extremely limited (CAA-agar: 5 g casamino acids, 1.18 g $K_2HPO_4.3H_2O$, 0.25 g $MgSO_4.7H_2O$, and 12 g agar L^{-1}, supplemented with 0.1 g human apo-transferrin and 1.68 g $NaHCO_3$ L^{-1} for additional iron chelation). We set up density treatments as follows: six replicate samples of the master culture were serially diluted in M9 buffer to obtain the following dilutions: 1 in 10^6 (low density); 1 in 10^4 and 1 in 10^2 (high density). We spread 20 μl samples of these diluted cultures to 10-cm-diameter plates of 25 mL CAA-agar and set these to incubate at 37°C.

After 48 h we scraped all bacterial growth from the surface of each plate with a sterile spatula and resuspended it in 6 mL M9. We vortexed and serially diluted these resuspended cultures, then plated them onto Kings B agar. We counted + and – CFUs on these plates after 24 h incubation at 37°C, and in this way we obtained data on change in relative proportions of + and – CFUs across the competition period. We fully randomized labeling, spreading, and counting of plates at each stage to minimize order effects.

INVESTIGATING THE CAUSAL MECHANISMS UNDERLYING DENSITY-DEPENDENT CHEAT FITNESS

We carried out a separate experiment to investigate the causal mechanism underlying patterns of density-dependent cheat fitness. Specifically, we tested whether the growth of cheat colonies varied with their proximity to cooperator colonies. First, we cultured strains overnight in Kings B medium then diluted them such that 1 μl of culture contained approximately 25 bacteria. Next, we set up 3 × 8 point lattices of 1 μl droplets of – culture on iron-limited agar (CAA, as above), by transferring culture from a microtitre plate using a 96-pin steel replicator tool (Boekel Scientific, Feasterville, PA). Each lattice of – droplets was bounded along one edge by an adjacent row of 8 × 1 μl droplets of a "neighbor" culture, at a density of approximately 600 cells per droplet. In this manner, we obtained 36 lattices (3 strain pairs × 3 treatments × 4 replicates), each of which comprised three rows of eight cheat colonies, growing on iron-limited agar, at varying distances from the row of eight "neighbor" colonies (either +, − or M9 buffer, "0"; see Fig. 1). We incubated plates at 37°C for 48 h and assayed the relative growth of cheat colonies in the lattices. First, we excised all colonies in each row, using a sterile 4-mm-diameter hole-punch, and transferred the cores to an eppendorf containing 0.5 mL M9 buffer. We thoroughly vortexed each eppendorf to wash cells off the agar and into suspension, and then, taking two replicate 200 μl samples of diluent (minus agar), we estimated the cell density of the resuspended cultures by measuring, with a spectrophotometer (M2 plate reader, Molecular Devices, Sunnyvale, CA), their optical absorbance at 600 nm.

STATISTICAL ANALYSES

To analyze the first set of experiments, we first calculated relative cheat fitness (v), by comparing the frequency of cheats at the beginning and end of each experiment. Specifically, v is given by $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_1 is the initial proportion of cheats in the population, x_2 is their final proportion

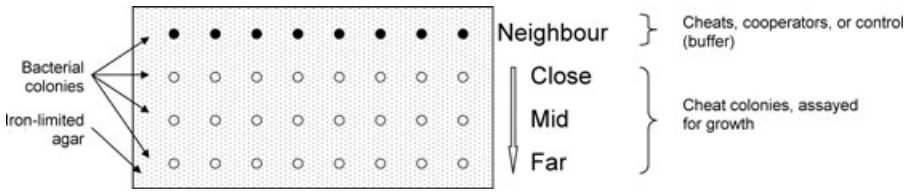

Figure 1. Design of experiment to demonstrate that physical proximity to cooperators is the causal mechanism underlying density dependence of cheat fitness. Circles indicate position of bacterial colonies in the lattice. Colonies from the lower three rows were assayed for growth after 48 h.

(Ross-Gillespie et al. 2007). The value of v therefore signifies whether cheats increase in frequency ($v > 1$), decrease in frequency ($v < 1$), or remain at the same frequency ($v = 1$). We assessed the nature and strength of the relationship between v and population density (n), using linear mixed effects models and general linear models with n as the explanatory variable, along with strain pair and the interaction term in the case of the maximal model. In each case, v and n were first log-transformed to comply with the assumptions of parametric analyses. In addition, we calculated an alternative measure for the fitness of cheats relative to cooperators, w, which is the ratio of the two strains' respective Malthusian growth parameters—essentially, the ratio of the number of doublings achieved by each subpopulation (Lenski et al. 1991). As above, we constructed a GLM with w as the response variable, and n and strain pair as fixed explanatory terms (where both w and n were first log transformed).

To analyze our productivity versus proximity data, we first calculated the relative productivity for each sample as its optical density relative to the average optical density across all samples for that same strain pair. We then constructed a linear mixed effects model with relative productivity as the response term (log transformed) and the following fixed effects: strain pair (A, B, or C); neighbor (+, −, or 0); distance to neighbor (close, mid, or far), as well as all two-way interactions. Because data were collected for multiple levels of the proximity treatment from the same replicate agar plates, and were thus not independent of each other, we included plate as a random effect in the model. Analyses were performed using S-Plus 8.0 (TIBCO Software Inc, Palo Alto, CA).

Results

In general, we found that cheats fared better under conditions of high population density (Fig. 2). In a model including all three strain pairs, the relative fitness of cheats was significantly positively correlated with initial population density ($F_{1,46} = 87.40$; $P < 0.001$). However, there were significant differences in relative cheat fitness across strain pairs (main effect: $F_{2,46} = 200.39$; $P < 0.001$) and in the patterns of density dependence across strain pairs (interaction: $F_{2,46} = 38.96$; $P < 0.001$). Indeed, when analyzed separately, strain pair A (in contrast to pairs B and C) did not show significant density dependence of cheat fitness ($F_{1,16} = 0.49$; $P = 0.49$).

When we analyzed our data using the alternative measure of relative fitness (w), differences between strain pairs were more pronounced. Here, strain pair A showed negative density dependence ($F_{1,16} = 12.39$; $P = 0.02$), whereas pairs B and C both showed positive density dependence, as in the above analyses. Consequently, there was no overall main effect of initial density on w ($F_{1,46} = 0.10$; $P = 0.756$), but a strongly significant interaction between strain pair and initial density ($F_{2,46} = 29.02$; $P < 0.001$). Comparing the two strain pairs that did show positive density dependence, we found further differences, in terms of the intercepts of their density dependence functions: B− had relative fitness ≥ 1 across all treatments, whereas C− had lower fitness than C+ at low cell density (v and w both <1) but greater fitness at high density (v and w both > 1).

In support of the assumption of our model, cheats were most productive when they were growing in close physical proximity to colonies of cooperators. The minimal model showed that the relative productivity of cheats was strongly influenced by the types of colonies growing nearby ($F_{2,84} = 144.04$; $P < 0.001$) and by the proximity to these colonies ($F_{2,84} = 33.88$; $P < 0.001$), although the latter did vary in strength across the three strain pairs ($F_{4,84} = 7.47$; $P < 0.001$). The effects of treatment and proximity, and the significant interaction between them ($F_{4,84} = 46.19$; $P < 0.001$), are illustrated in Fig. 3. There was no significant main effect of strain pair ($F_{2,9} = 1.49$; $P = 0.275$).

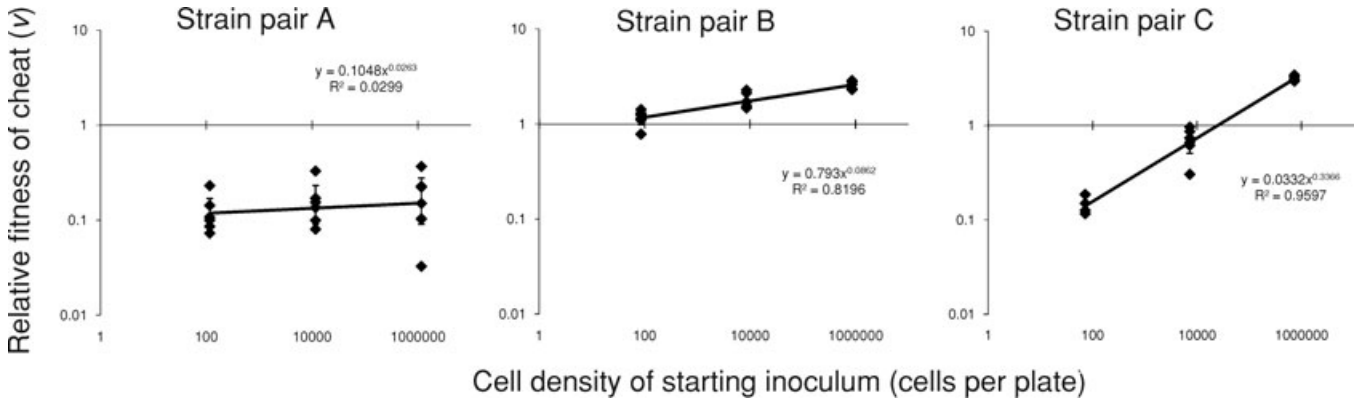

Figure 2. Positive density dependence of cheat relative fitness in three independently derived strain pairs (A–C). Each datapoint represents a different mixed population of cooperator and cheat bacteria cultured together on an agar plate for 48 h.

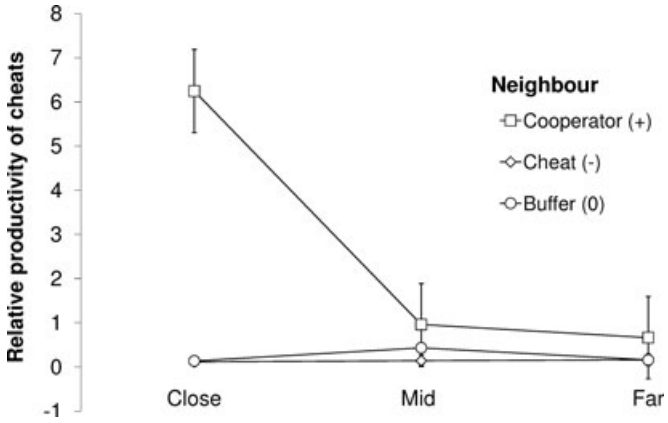

Figure 3. **Interaction plot of fitted mean values from our analyses, showing how the productivity of cheat colonies depends on whether or not cooperators are growing in the vicinity, and how close they are to these cooperators. Error bars show one standard error around the means.**

Discussion

We have investigated, theoretically and empirically the conditions under which cooperation in microbes is subject to density-dependent selection. We have shown in our theoretical models that, when considering the production of public goods, cheats are predicted to have a higher relative fitness at higher population densities (positive density dependence), but that extent of density dependence will be weaker in more structured populations (Fig. 4A). In contrast, when considering prudent use of limited resources (tragedy of the commons), cheats can have either a higher or a lower relative fitness at higher population densities (positive or negative density dependence; Fig. 4B), depending upon the biological details. We then examined experimentally the form of cooperation for which our models showed a clear a priori prediction could be made—the production of public goods. We tested this prediction using the production of iron-scavenging siderophore molecules in *P. aeruginosa* as a model trait. As predicted, we found that cheats have higher fitness at greater population densities (Fig. 2). Furthermore, we found support for the underlying assumption leading to this prediction, that cheats are better able to exploit the cooperative siderophore production of others, when they are physically closer to them (Fig. 3).

We predicted that the direction of density-dependent selection on cooperation depends first upon the form of cooperation (public good production or self-restraint in resource use) but also upon biological details. Considering public goods, an increase in cell density will allow cheating cells a greater access to the public goods produced by its cooperative neighbors. Another way of thinking about this is that an increase in density increases the number of cells that each individual can interact with, and hence tend to decrease the relatedness of an individual to all the individuals in the group, including itself ("whole-group" relatedness; Pepper 2000). The net effect is an increase in the relative fitness of cheats at greater population densities. Support for this prediction has been provided by Greig and Travisano (2004) in relation to invertase production in yeast, and this study in relation to siderophores in bacteria (Fig. 2). In both of these cases, a clear prediction could be made because relatedness to other cells, r, did not vary with cell density (i.e., the proportion of cheats was held constant in the starting inoculum).

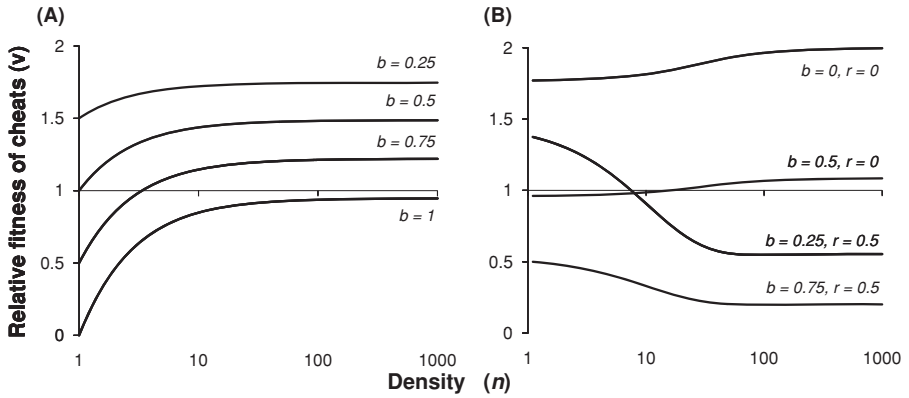

Figure 4. **Model demonstrating how the fitness of cheats relative to cooperators can be subject to density dependence. Under public goods cooperation (A), cheat relative fitness, v, is positively related to density across most of the parameter space but is highly sensitive to b, a parameter describing how beneficial cooperation is to a recipient. For this illustration, we set $a = 1$ and $c = r = 0.5$. Here, n describes the total number of cells interacting within a social neighborhood. We assume that cooperators invest $X = 1$ into public goods and cheats invest $X = 0$ and, mirroring the experiments, that cheats make up 10% of the population. Under self-restraint cooperation (B), cheat relative fitness can be either positive or negatively density dependent, depending on the values of both b (see above) and r, relatedness to other cells in the social group. For this illustration, we set $a = k = 0.25$, assume cooperators show self-restraint $X = 0.5$ and cheats $X = 0$, and that cheats make up 10% of the population.**

Another potential factor that could affect the density dependence of cooperation is that, over a fixed period, greater absolute population growth can occur in populations that start from lower inoculum densities. Such populations consequently undergo more rounds of growth in which cheats can exploit cooperators (see Ross-Gillespie et al. 2007). This should, if anything, enhance the relative fitness of cheats at low population densities (negative density dependence), so the fact that, in our experiments, we observed a clear overall pattern of positive density dependence despite this potential confounding effect, demonstrates the strength of the effect of density per se. Greig and Travisano (2004) suggested that fluctuating selection for increased then decreased cooperation, as yeast cells colonize new patches and then grow to high densities, could allow for the maintenance of both cooperators and cheats in the population (polymorphism). More generally, we might expect microbes to facultatively adjust their cooperation strategies depending on local cell densities, for example through the use of quorum sensing systems (Brown and Johnstone 2001; Keller and Surette 2006; Diggle et al. 2007a, b).

In contrast, when considering cooperative self-restraint in resource uptake, the relative fitness of cheats, which have a higher uptake rate and less-efficient metabolism of resources, can either increase or decrease with host density, depending upon biological details (parameter values). Although the advantage of more-efficient metabolism of resources is greater when cell density is high enough for resources to be growth-limiting, the individual incentive for such cooperation can decrease with increasing cell density because the increasing number of social partners allows cheats to exploit cooperators (lower whole-group relatedness). These two effects are in opposition and so cheats that use resources quickly and inefficiently may be increasingly favored or disfavored as cell density increases, depending upon which of the two effects is strongest. More generally, this emphasizes that, although the public goods dilemma and the tragedy of the commons (self-restraint) are both problems of cooperation and can potentially be viewed as alternate forms of the same problem (Rankin et al. 2007), they are not strictly symmetrical and can lead to very different predictions (Dionisio and Gordo 2006).

The prediction of cheats having a lower fitness at higher densities (negative density dependence) has previously been made and supported in studies of the competition between yeast strains following alternative metabolic strategies (Pfeiffer and Bonhoeffer 2003; MacLean and Gudelj 2006; MacLean 2008). In yeast, respirers (cooperators) use resources slowly but with a higher yield of ATP than respiro-fermenters (cheats), which enjoy fast resource use but with a much smaller ATP yield. Although the observed negative density dependence of cheat fitness in yeast may occur for the reason discussed above, an added complication in this system is that the metabolic pathway employed by cheats yields toxic byproducts that decrease fitness as they accumulate, and this accumulation of toxins is increased at higher population densities (MacLean and Gudelj 2006). Thus, one consequence of toxin accumulation in this system is that it increases the parameter space over which negative density dependence is expected. Another potential complication that we have not considered here is the possibility of interactions between self-restraint and public goods cooperation, when they are linked (MacLean 2008).

Patterns of density-dependent fitness are intrinsically sensitive to the measure of fitness used. Our preferred measure of relative fitness (v, see above) captures the relative change in frequency of a genotype over time and, as such, is compatible with the population genetics concept of fitness. However, although a change in genotype frequency over time can indeed reflect different rates of growth or persistence among genotypes within a given population, this measure can be problematic in between-population comparisons if the extrinsic conditions (i.e., treatments) afford different populations different opportunities for growth. When resources are finite and fixed (i.e., batch culture, as in our experiment), populations starting from low density will complete more generations during the observation period because they start growing at a density far below carrying capacity. In contrast, at high density a population will undergo little such exponential growth before reaching stationary phase. Thus, in the low inoculum scenario, absolute fitness differences between competing strains could be magnified relative to what would be seen in populations starting from high-density inocula, resulting in density-dependent fitness effects—that may have nothing to do with social interactions among strains. We investigated this possibility by also considering an alternative fitness measure that controls for differences in the number of generations (w, see above). Analyses of strain pairs B and C yielded qualitatively similar results for both fitness measures, confirming that the density-dependent fitness patterns we observed in these strains (Fig. 2) cannot be explained by this confounding generation effect only.

Our experimental data on siderophore production in *P. aeruginosa* provided support for the prediction of our public goods model in two of the three independent strain pairs we tested. In these two cases, relative cheat fitness was positively correlated with population density (Fig. 2). Across all three of the pairs, however, there was considerable variation in the intercepts of the density-dependent fitness function. One cheat strain consistently underperformed its cooperator progenitor (pair A) under our experimental conditions; another showed greater relative fitness at all densities tested (pair B); whereas in the third, experimentally evolved pair, cheats outperformed cooperators at high density but lost the advantage at low density (pair C). The strain pairs may differ for a number of reasons, and so we cannot determine the exact cause of this variation. One possibility is that our cheat strains A and C could be acting as cheats not just for siderophore production, but for other traits too. For example, our pair A cheat

may, under certain conditions, also act as a cheat in the context of quorum sensing (S. P. Diggle and N. Jiricny unpubl. data). This highlights the value, when studying the fitness consequences of a given trait, of testing multiple, independently derived bacterial strains (Velicer et al. 2000; Ross-Gillespie et al. 2007). Gene deletions, for instance, could give rise to unforeseen pleiotropic effects, whereas artificial selection against a given trait could generate more "natural" mutants, with compensatory adaptations to counteract potential negative pleiotropic effects associated with the loss of the target trait.

Although we have focused on microbes, our predictions could also apply to other taxa. The importance of density dependence in the production of public goods has also been much discussed for humans, in the economics literature. Since Olson (1965), a number of economics studies of public goods dilemmas have noted how cooperative behavior frequently decreases and "free-rider" behavior increases within larger groups (e.g., Chamberlin 1974; Andreoni 1988; Stonebraker 1993; Gaube 2006; Alencar et al. 2008). However, as is often the case with human behavior, even small changes in experimental design or context can have important effects. For instance, Isaac and Walker (1988) and Isaac et al. (1994) showed that when the marginal incentives to individuals were scaled to remain constant across different group sizes, free-riding behavior actually decreases at higher densities. More generally, for any organisms that live at high density, cooperation will be most favored when interactions take place between close relatives (i.e., cooperators interact almost exclusively with other cooperators; Hamilton 1964). A common way to achieve this is to have relatively low dispersal into or out of the population—a pattern that has been observed in both microbes (e.g., biofilms, where cells encase themselves in a viscous matrix; Xavier and Foster 2007) and metazoans, including social invertebrates (Hamilton 1972; Bilde et al. 2005; Peer and Taborsky 2007) and molerats (Jarvis et al. 1994).

Although we conducted our experiments in an artificial setting, cooperator-cheat dynamics in natural microbial populations are also likely to show density dependence. A major task for the future will be to understand the role of such dynamics in the context of pathogen infections, where the production of cooperative public goods determines virulence (West and Buckling 2003; Harrison et al. 2006; Rumbaugh et al. 2009). Possible complications in more natural systems could include (1) interactions between density- and frequency-dependent effects, and (2) additional density-dependent effects in the case of public goods where production is regulated by quorum sensing mechanisms (Brown and Johnstone 2001).

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Supporting Information

The following supporting information is available for this article:

Appendix S1. Demonstration of the density dependence of cheat relative fitness in the public goods and self-restraint cooperative scenarios.

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).

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Additional results and discussion can be found in a document at http://www.repository.naturalis.nl/record/289893.

Density dependence and cooperation: theory and a test with bacteria

Ross-Gillespie et al.

Supplementary Information: Demonstration of the density dependence of cheat relative fitness in the public goods and self-restraint cooperative scenarios

Public goods

From equations (2) & (3), we find that the relative fitness of cheats in the public goods cooperation model is given by:

$$v = 1 + \frac{c}{1-cz}\delta x - \frac{abz^{a-1}}{1-b+bz^a}\left(\frac{1}{n} + \frac{n-1}{n}r\right)\delta x + O\left(\delta^2\right), \quad \text{(A1)}$$

where z is the population average level of cooperation. Differentiating the RHS of equation (A1) by n reveals the density dependence:

$$\frac{\mathrm{d}v}{\mathrm{d}n} = \frac{abz^{a-1}}{1-b+bz^a}\left(\frac{1}{n^2}(1-r) - \frac{n-1}{n}\frac{\mathrm{d}r}{\mathrm{d}n}\right)\delta x, \quad \text{(A2)}$$

to first order in δx. A sufficient condition for the RHS of equation (A2) to be positive is $r < 1$ and $\mathrm{d}r/\mathrm{d}n \leq 0$: less than clonal within-group relatedness, that does not increase with cell density, ensures that the relative fitness of cheats is an increasing function of cell density (positive density dependence). Note that if $r = 1$ at all cell densities (and hence $\mathrm{d}r/\mathrm{d}n = 0$), then the RHS of equation (A2) is zero, and hence there is no density dependence in a population that is structured into clonal social groups. More generally, if we express the others-only relatedness as a function $r = f(n) + g$ of cell

density, where f is an arbitrary function and g is an arbitrary constant, then the impact of raising others-only relatedness uniformly over all cell densities is given by:

$$\frac{\mathrm{d}}{\mathrm{d}g}\left(\frac{\mathrm{d}v}{\mathrm{d}n}\right) = -\frac{abz^{a-1}}{1-b+bz^{a}}\frac{1}{n^{2}}\delta x, \tag{A3}$$

which is negative. Hence, as the population becomes increasingly genetically structured, the density dependence of cheat relative fitness decreases.

We now consider the possibility that, at low cell density, not all public goods end up being used by bacterial cells. For simplicity, we assume that the probability of contact between a given public good molecule and a given cell is constant through time, i.e. a constant per cell per molecule per unit time rate of interaction (k). The probability that a given molecule encounters no bacterial cells in the relevant time period is then exp(-kn), and so the proportion of public goods molecules that are ultimately used is 1-exp(-kn). Thus, there are nY(1-exp(-kn)) public goods molecules used up locally, and the focal cell receives a proportion 1/n of these. Hence, while in the main text we assumed that each cell received an amount Y of public good molecules, it is more realistic to assume that they receive Y(1-exp(-kn)). Implementing this added realism, fitness function (3) is now given by:

$$W\left(X,Y\right) = \left(1-b+b\left(Y\left(1-\exp\left(-kn\right)\right)\right)^{a}\right)\left(1-cX\right). \tag{A4}$$

Following the usual procedure, we obtain a relative cheat fitness of:

$$v = 1 + \frac{c}{1-cz}\delta x - \frac{abz^{a-1}\left(1-\exp\left(-kn\right)\right)^{a}}{1-b+b\left(z\left(1-\exp\left(-kn\right)\right)\right)^{a}} R\delta x + O\left(\delta^{2}\right), \tag{A5}$$

and, differentiating the RHS of expression (A5) by n reveals the density dependence:

$$\frac{\mathrm{d}v}{\mathrm{d}n} = \frac{abz^{a-1}\left(1-\exp\left(-kn\right)\right)^{a}}{1-b+b\left(z\left(1-\exp\left(-kn\right)\right)\right)^{a}} \times\left(\frac{1-r}{n^{2}} - \frac{a(1-b)k}{1-b+b\left(z\left(1-\exp\left(-kn\right)\right)\right)^{a}} \frac{\exp\left(-kn\right)}{1-\exp\left(-kn\right)}\left(\frac{1}{n}+\frac{n-1}{n}r\right) - \frac{n-1}{n}\frac{\mathrm{d}r}{\mathrm{d}n}\right)\delta x, \tag{A6}$$

to first order in δx. Assuming $\mathrm{d}r/\mathrm{d}n \le 0$, i.e. the others-only relatedness of social partners does not increase with cell density, then a sufficient condition for the relative fitness of cheats to increase with population density (positive density dependence, $\mathrm{d}v/\mathrm{d}n > 0$) is:

$$b\left(\frac{ak\exp\left(-kn\right)}{1-\exp\left(-kn\right)}\left(\frac{1}{n}+\frac{n-1}{n}r\right) - \left(1-\left(z\left(1-\exp\left(-kn\right)\right)\right)^{a}\right)\frac{1-r}{n^{2}}\right) > \frac{ak\exp\left(-kn\right)}{1-\exp\left(-kn\right)}\left(\frac{1}{n}+\frac{n-1}{n}r\right) - \frac{1-r}{n^{2}} \tag{A7}$$

We now demonstrate that this is satisfied for sufficiently large values of b, and that there always exists a $b \le 1$ that is large enough to satisfy the condition. First, if the RHS of condition (A7) is positive, then the condition can be rearranged into the form $b > b^{*}$, where:

$$b^* = \frac{ak\dfrac{\exp(-kn)}{1-\exp(-kn)}\left(\dfrac{1}{n}+\dfrac{n-1}{n}r\right)-\dfrac{1-r}{n^2}}{ak\dfrac{\exp(-kn)}{1-\exp(-kn)}\left(\dfrac{1}{n}+\dfrac{n-1}{n}r\right)-\dfrac{1-r}{n^2}+\left(z\left(1-\exp(-kn)\right)\right)^a\dfrac{1-r}{n^2}} \quad \text{(A8)}$$

lies in the range $0 < b^* < 1$. Alternatively, if the RHS of condition (A7) is negative then, if the LHS is also negative, we obtain the condition $b < b^*$ where $b^* > 1$, and if the LHS is positive we obtain the condition $b > b^*$ where $b^* < 0$; in either scenario, the condition is satisfied irrespective of the value of b. In summary, so long as public goods are sufficiently beneficial (large enough b), the relative fitness of cheats is never a decreasing function of cell density. For example, if the public good is necessary for growth ($b = 1$), then any density dependence in the relative fitness of cheats will be positive.

As before, the impact of relatedness upon density dependence is found by first making the substitution $r = f(n) + g$, then differentiating the RHS of (A6) with respect to g, to obtain:

$$\frac{\mathrm{d}}{\mathrm{d}g}\left(\frac{\mathrm{d}v}{\mathrm{d}n}\right) = -\frac{abz^{a-1}\left(1-\exp(-kn)\right)^a}{1-b+b\left(z\left(1-\exp(-kn)\right)\right)^a}\left(\frac{1}{n^2}+\frac{a(1-b)k}{1-b+b\left(z\left(1-\exp(-kn)\right)\right)^a}\frac{\exp(-kn)}{1-\exp(-kn)}\frac{n-1}{n}\right)\delta x, \quad \text{(A9)}$$

which is always negative.

Self-restraint

From equations (2) & (4), we can write the relative fitness of cheats as:

$$v = 1 + \left(\frac{1}{1-z} - \frac{abz^{a-1}}{1-b+bz^a} + \frac{1}{1-z}\left(kn(1-z)\frac{\exp(-kn(1-z))}{1-\exp(-kn(1-z))} - 1 \right) R \right) \delta x + O(\delta^2), \quad \text{(A10)}$$

where z is the population average level of self restraint. Differentiating with respect to cell density n gives us:

$$\frac{\mathrm{d}v}{\mathrm{d}n} = \left(k\frac{\exp(kn(1-z))(1-kn(1-z))-1}{(\exp(kn(1-z))-1)^2} R + \frac{1}{1-z}\left(kn(1-z)\frac{\exp(-kn(1-z))}{1-\exp(-kn(1-z))} - 1 \right) \frac{\mathrm{d}R}{\mathrm{d}n} \right) \delta x, \quad \text{(A11)}$$

to first order in δx. This RHS is of the form $(\alpha+\beta)\delta x$, where $\alpha < 0$ and $\beta > 0$ (assuming $\mathrm{d}R/\mathrm{d}n < 0$), and hence depending on the relative magnitudes of these two quantities, the relative fitness of cheats may be positively or negatively density dependent. The negative term α reflects how increasing cell density makes resource availability increasingly limited, which relatively favours cooperative self restraint to make the best use of those resources. However, the positive term β reflects how increasing cell density leads to decreasing whole-group relatedness, which relatively favours cheats who strive for more than their fair share of group resources. Moreover, at low cell density (relative to resource availability; $kn(1-z)\rightarrow 0$), the relative fitness of cheats approaches:

$$v = 1 + \left(\frac{1}{1-z} - \frac{abz^{a-1}}{1-b+bz^a} \right) \delta x + O(\delta^2), \quad \text{(A12)}$$

i.e. the kin-selection effects mediated through resource availability become negligible, and cheats are favoured over cooperators provided that the efficiency benefit of prudent resource use is sufficiently low, i.e.:

$$b < \frac{1}{1 - z^{a-1}\left(z - (1-z)a\right)}. \qquad \text{(A13)}$$