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Understanding microbial cooperation

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ABSTRACT

The field of microbial cooperation has grown enormously over the last decade, leading to improved experimental techniques and a growing awareness of collective behavior in microbes. Unfortunately, many of our theoretical tools and concepts for understanding cooperation fail to take into account the peculiarities of the microbial world, namely strong selection strengths, unique population structure, and non-linear dynamics. Worse yet, common verbal arguments are often far removed from the math involved, leading to confusion and mistakes. Here, we review the general mathematical forms of Price's equation, Hamilton's rule, and multilevel selection as they are applied to microbes and provide some intuition on these otherwise abstract formulas. However, these sometimes overly general equations can lack specificity and predictive power, ultimately forcing us to advocate for more direct modeling techniques.

1. Introduction

Cooperation presents a fundamental challenge to customary evolutionary thinking. If only the fittest organisms survive, why would an individual ever pay a fitness cost for another organism to benefit? Traditionally, kin selection and group selection have been the most prominent explanations for the maintenance of cooperation in nature. Kin selection refers to cooperative behaviors being favored when they are preferentially directed towards relatives (Hamilton, 1964). In turn, group selection, also known as multilevel selection, suggests that altruistic traits can be favored because of their beneficial effect on a group, despite the individual cost of such behaviors (Wilson, 1975).

While the connection between kin and multilevel selection was initially unclear, recent theoretical work has elucidated many of the similarities and differences between the two concepts (Nowak, 2006b; Page and Nowak, 2002; Fletcher and Zwick, 2006; Wenseleers et al., 2009). In particular, the underlying theme behind all mechanisms for the evolution of altruism is the assortment of similar individuals (Fletcher and Doebeli, 2009). When similar individuals are assorted, cooperators are more likely than average to interact with other cooperators and non-cooperating defectors are, in turn, more likely to interact with defectors. This assortment is ascribed to relatedness in kin selection and between-group variance in multilevel selection models. Both methods are equivalent when applied correctly and under certain assumptions. Unfortunately, when these assumptions do not hold, both methods resort to abstract generalities, making application difficult and prone to error.

Microbes present a unique opportunity for scientists interested in the evolution of cooperation because of their well-characterized and simple genetics, fast generation times, and easily manipulated and measured interactions. While these advantages are often well appreciated, other differences between organisms of the microscopic and macroscopic world are sometimes forgotten when transferring ideas and methods from the study of animals to that of microbes. Important differences include strong selection strengths, fast evolution times, high levels of diversity, and non-linear dynamics, all of which invalidate many less general techniques derived using specific assumptions, a fact too often ignored or simply unknown by non-theorists.

In this review, we examine the standard techniques used to understand cooperation, as they are applied to microbes. This allows us to make several simplifications, particularly in genetics, but it also means that we will not cover any technique inappropriate to microbes. With that said, many of our points are pertinent to any scientist in the field of cooperation or microbial biology, but some may only be valid when considering microbial cooperation.

2. Four basic classes of cooperation

To begin, let us define cooperation as any act that increases the fitness of others. Now, if we have two strains of a microbe, one an obligate cooperator and the other a defector that never cooperates, there are still four fundamentally different classes of interactions that fit within this definition of cooperation. For

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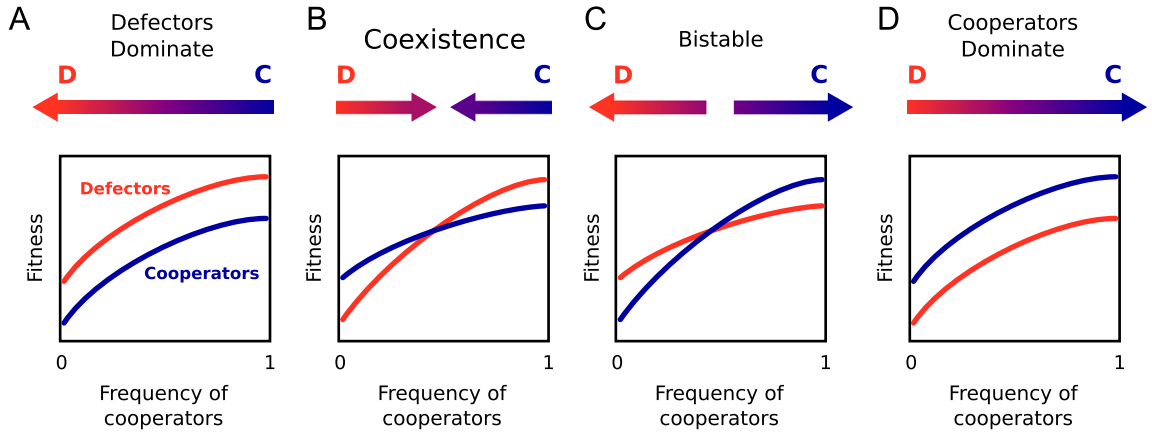

Fig. 1. (Color online) There are four basic, distinct outcomes of a cooperative interaction. (A) Defectors (red) may always have a higher fitness than cooperators (blue), which would lead to the extinction of cooperators. (B) Alternatively, cooperators may have a higher fitness than defectors when they make up a small fraction of the population and a lower fitness at high fractions, leading to coexistence of cooperators and defectors. (C) Also, cooperators may have a higher fitness only when they make up a large fraction of the population and a lower fitness otherwise. This creates an unstable fixed point, above which cooperators fix in the population and below which defectors fix, characteristic of bistability. (D) Finally, cooperators may always have a higher fitness than defectors (or a complicated combination of (B) and (C)). In game theory, the popular names for these interactions (when linear) are (A) prisoner's dilemma, (B) snowdrift, (C) stag hunt, and (D) mutually beneficial or harmony game.

example, imagine mixing the two strains at different relative fractions in a test tube, either: one strain performs better than the other at every frequency, or one strain grows faster than the other at some fractions and slower at other fractions (Fig. 1). If one strain always does better – either the cooperator or defector – then we say that it dominates and natural selection will always favor the increase in frequency of the dominating strain (Fig. 1A and D). Thus, if we grow the cells in a turbidostat where the conditions and total population size are kept constant by continuous dilution, then the dominated strain will eventually go extinct—barring any stochastic effects which we will ignore here.

If, however, one strain does not always grow faster than the other, predicting the final result of such a experiment will be slightly more complicated. For instance, if cooperators grow better at low frequencies and worse at high frequencies, then the two strains will coexist at some intermediate frequency, independent of the starting frequency of the experiment (Fig. 1B). On the other hand, if cooperators grow better at high frequencies and worse at low frequencies, then cooperators will fix in the population if they start at a high enough frequency, but will go extinct if they start at a lower frequency (Fig. 1C).

For most cooperative interactions there are thus only four possible outcomes: either (A) defectors will always fix in the population, (B) the two strains will coexist at some intermediate frequency, (C) one strain will fix depending the initial frequency, or (D) cooperators will always fix (although there may be complications, e.g. see MacLean et al., 2010). Each situation yields drastically different dynamics and should be treated as a distinct circumstance. When cooperators dominate defectors, natural selection acts to increase the mean fitness of the population, the socially optimal outcome. On the other hand, if defectors dominate cooperators, the mean fitness decreases and we are often interested in how this helpful cooperative behavior can be maintained despite this dominance, a question kin and group selection were developed to answer. Before discussing these theories, we will first examine in more detail the outcome of the two other forms of cooperation, both of which are limited by defectors, but not dominated by them.

While modelers often assume that any costly behavior displayed by bacteria that helps other cells is dominated by a defector cell that does not display that behavior, this is actually rarely the case. In fact, coexistence between cooperators and defectors has been found both in nature and the lab for many cooperative systems, including protease and siderophore production, β-lactam antibiotic degradation, defective viruses, fruiting body formation, and sucrose metabolism in yeast (Diggle et al., 2007; Ross-Gillespie et al., 2007; Dugatkin et al., 2005; Turner and Chao, 2003; smith et al., 2010; Gore et al., 2009). These traits are said to have negative frequency-dependence because the relative fitness of a cooperator decreases as their frequency increases. In particular, the stable mixed equilibrium is where the relative fitness equals one. A common theme among these negative frequency-dependent traits is that a cooperator somehow receives a more than average share of the public good that it personally produces. A prime example of this is the cooperative sucrose metabolism system in budding yeast, *Saccharomyces cerevisiae* (Greig and Travisano, 2004; Gore et al., 2009; MacLean et al., 2010).

S. cerevisiae cannot import the sugar sucrose directly into the cell very efficiently. Instead, yeast is forced to break the disaccharide sucrose into glucose and fructose outside of the cell with the extracellular enzyme invertase and then import the monosaccharides glucose and fructose. Unfortunately for yeast, because this happens in the periplasmic space, approximately 99% of the created glucose (and fructose) diffuses away to its neighbors and the cell can only capture about 1% of the products of sucrose hydrolysis (the capture efficiency, ε, is thus 0.01; Gore et al., 2009). This would suggest that a cheater strain that does not pay the cost of producing invertase could potentially take advantage of the invertase producing cooperators if the cost is higher than the benefit of the extra 1% of glucose captured. In a simple model, where growth is a linear function of the available glucose (Fig. 2B), cooperators would either always do better or always do worse than defectors depending on whether or not the normalized cost, c, is greater than ε. However, experimental results suggest coexistence between cooperators and defectors (Fig. 2A); therefore, one of the assumptions of the simple linear model is incorrect.

As it turns out, the growth rate is actually a concave function of the available glucose, which leads to coexistence of cooperators and defectors because there are diminishing returns to increased glucose (Fig. 2C). Thus, when the local glucose concentration is low, the increased glucose due to invertase production outweighs the cost of cooperation, but as there is more glucose in the environment from other cooperators, the additional growth rate per glucose molecule reduces below the cost of invertase production. This qualitative model has proven to be robust to experimental manipulation of the costs and benefits of cooperation and

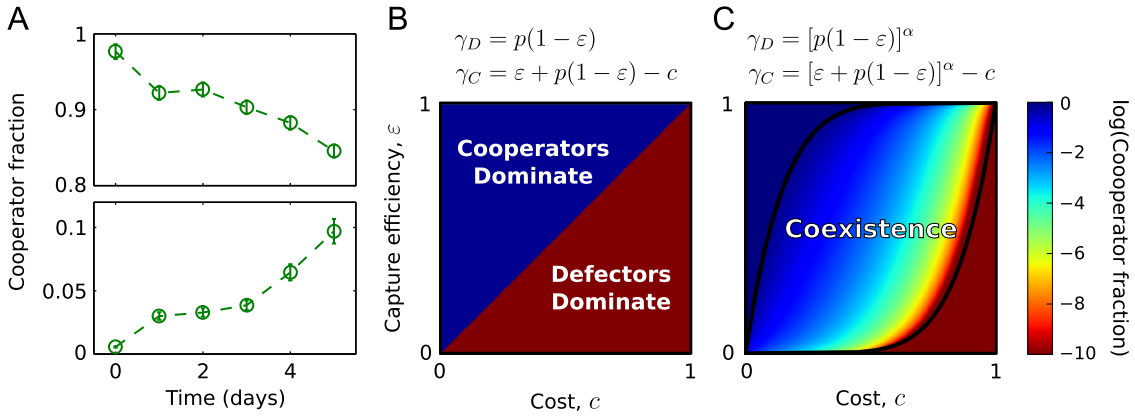

Fig. 2. Non-linear dynamics can lead to coexistence of cooperators and defectors. (A) Cooperators and defectors coexist in the cooperative sucrose metabolism system, which can be seen by their mutual invasability, where cooperators can invade pure defector cultures (bottom) and defectors can invade pure cooperator cultures (top). (B) In a simple model of the cooperative yeast sucrose metabolism system, if the normalized growth rate, γ, is a linear function of the available glucose, then cooperators or defectors always dominate depending on the parameters. Here, we assume that cooperators pay a cost, c, and capture a small fraction, ε, of the glucose made personally, with the rest, $1-\varepsilon$, diffusing away to be used by other cells. The amount of available glucose to a cell is then approximately given by $\varepsilon+p(1-\varepsilon)$, where p is the proportion of cooperators in the population. Thus cooperators always grow faster than defectors if $c<\varepsilon$ and grow slower otherwise. (C) If, however, the growth rate is a non-linear function of the available glucose, then there is a large region where coexistence between cooperators and defectors is measurable (between dark lines). The exponent, α, was experimentally measured to be 0.15 ± 0.01 (adapted from Gore et al. (2009)).

provides insight into how non-linear dynamics can lead to coexistence (see Gore et al., 2009, for details).

In contrast to deterministic coexistence, some systems may instead show bistability, which is characterized by mutual non-invasability: cooperators cannot invade a pure defector culture, but also defectors cannot invade a pure cooperator culture. Bistability is predicted whenever cooperators preferentially interact with themselves. Perhaps the most well-known example of this is the hypothetical "green beard" gene (Hamilton, 1964; Dawkins, 1976). Imagine a gene that simultaneously codes for a green beard – or any other arbitrary tag – and a cooperative behavior directed towards individuals with green beards. Such a green beard gene could have an advantage and spread in the population. However, if the cost of this gene is too high, then it may still be disfavored at low frequencies because it does not receive enough benefit. Alternatively, two different versions of a green beard gene may compete and could also show bistability. Despite the theoretical appeal, green beard genes are rare in nature, mostly because it is difficult for a single gene to code for a "beard," a recognition mechanism, and a cooperative action. With that said, the *flo1* flocculation gene in yeast has recently been identified as a green beard gene by causing bearers of the gene to stick together, protecting the inside of the clump from harm, like the build up of ethanol in the environment (Smukalla et al., 2008). Also, the *csaA* self adhesion gene in the slime mold *Dictyostelium discoideum* works by a similar mechanism to exclude non-bearers from fruiting bodies (Queller et al., 2003). Although it is out of the scope of this paper, mutually antagonistic relationships can also show bistability (Majeed et al., 2011).

If, however, cooperators always grow slower than the defectors that they are mixed with, then an additional mechanism is required to maintain cooperation. This situation corresponds to cooperators paying some cost, not necessarily a fixed amount, for others to benefit in some way. While in the well-mixed case, cooperators are destined for extinction, the dynamics become more complicated when structure is added to the population. Now, if cooperators on average gain a high enough proportion of the benefits from other cooperators by interacting with them more often than defectors do, then the increased benefits may outweigh the costs of cooperation and they can spread in the population. Thus, the simplest and most fundamental explanation for the maintenance of costly cooperation is that cooperators must interact more often with cooperators than defectors do. This assortment of similar individuals (i.e. cooperators with cooperators and defectors with defectors) is the ultimate outcome of every mechanism for the evolution of otherwise dominated cooperation, including kin discrimination, group selection, direct and indirect reciprocity, spatial structure and many others (Axelrod and Hamilton, 1981; Nowak and Sigmund, 2005; Ohtsuki et al., 2006; Nowak, 2006b; Fletcher and Zwick, 2006, 2007; Taylor and Nowak, 2007).

In the next section we introduce Price's equation, a powerful and visually appealing tool that connects many kin and multilevel selection models, particularly when usual simplifications are impossible. After introducing Price's equation, we provide some intuition and discuss some of its limitations; any reader already very comfortable with Price's equation can skip to Section 4 on Hamilton's rule.

3. Price's equation

The Price equation provides a general and exact mathematical description of evolution, with applications in kin and multilevel selection models (Price, 1970; Page and Nowak, 2002). First assume each individual, i, has a genotype, G_i, that can be quantitatively described. For example, G_i is often arbitrarily set to 1 if the (haploid) individual has the cooperative gene and 0 if it does not, a convention we will repeat in this paper. Now, if we want to know how the genotype frequency changes in the population over time, we first need to define an individual's fitness, W_i, to be the number of i individuals at the next time point divided by the number present now. For example, the two time points could be in generation times, in which case W_i would be a measure of how many offspring an individual has. The change in average genotype in the population, $\Delta\overline{G}$, is then given by the Price equation:

$$\overline{W}\Delta\overline{G} = \mathrm{Cov}(W,G) + \overline{W_i \Delta G_i} \tag{1}$$

where $\mathrm{Cov}(W,G)$ is the covariance between fitness and genotype in the population and $\overline{W_i \Delta G_i}$ accounts for the average change in genotype between parent and offspring (e.g. mutation). For asexual microbes, mutation and other effects that might change an offspring's genotype are often assumed to be small, in which

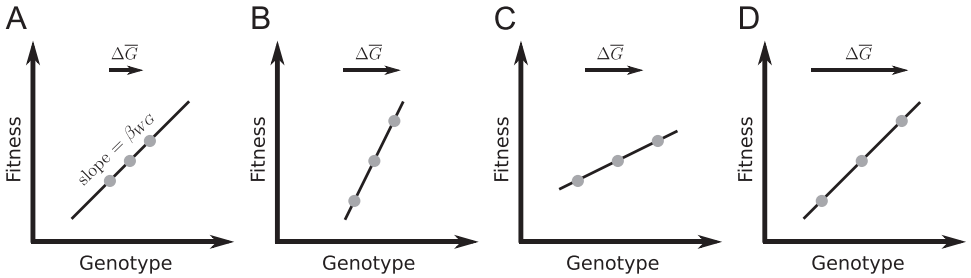

Fig. 3. Understanding how the Price equation works can aid in understanding evolution. (A) In its simplest form, Price's equation states that the change in genotype in a population is $\Delta\overline{G} = \mathrm{Cov}(W,G) = \beta_{WG}\mathrm{Var}(G)$, where W is relative fitness here. (B) Doubling the slope of the linear regression, β_{WG}, while keeping everything else constant, doubles $\Delta\overline{G}$. (C) Also note that increasing the spread of genotypes by a factor of 2 reduces β_{WG} by a factor of $\frac{1}{2}$, but it multiplies the variance by $2^2=4$ so $\Delta\overline{G}$ still doubles. (D) Effects multiply and combining (B) and (C) leads to a quadrupling of $\Delta\overline{G}$. For the purpose of illustration, the data points all sit perfectly on the regression, but that is not necessary for Price's equation to work.

case the Price equation becomes

$$\overline{W}\Delta\overline{G} = \mathrm{Cov}(W,G) = \beta_{WG}\mathrm{Var}(G) \tag{2}$$

where β_{WG} is the slope of the least-squares linear regression of fitness on genotype and Var(G) is the variance of genotypes in the population—the last equality being justified by the definition of covariance (Fig. 3). Increasing the variance or the average effect of genotype on fitness, β_{WG}, increases the speed of evolution. In fact, when fitness is perfectly heritable, Price's equation reduces to Fisher's Fundamental Theorem of Natural Selection which states

$$\Delta\overline{W} = \frac{\mathrm{Var}(W)}{\overline{W}} \tag{3}$$

which is valid when G is replaced with W ($\beta_{WW}=1$) and the last term in (1) is neglected. Price's equation is a simple and visually appealing formula that is widely used in the kin and multilevel selection literature; however, some of its limitations should be noted. First, practitioners often confuse probability theory and statistics when applying Price's equation which has consequences for its interpretation in derivations and models. Because this point is subtle and out of the scope of this review, we refer the interested reader to van Veelen (2005, 2009, 2010). On another technical note, the Price equation lacks dynamical sufficiency by only telling the current speed and direction of selection and not the path of evolution (Traulsen, 2010). Finally, with generality comes a loss of specificity, and Price's equation can mask many of the important intricacies of the system. This is a point we will elaborate on later because the same criticism holds for many kin selection and multilevel selection models that use Price's equation. While powerful and general, the Price equation does not usually ease empirical calculations and can only aid in understanding a social behavior when it is coupled with an explicit model. We will now move on to a generalized form of Hamilton's rule, a simple two variable model, that uses Price's equation in its derivation.

4. Hamilton's rule

In 1964, Hamilton stated his famous rule: a cooperative act will be favored by natural selection if the cost, c, of performing the cooperative act is less than the benefit, b, given to the other individual times the relatedness, r, between the two individuals, or equivalently if

$$rb-c>0. \tag{4}$$

While pleasingly simple, the assumptions that went into deriving this rule almost never hold even in simple models, much less microbial systems (Cavalli-Sforza and Feldman, 1978; Karlin and Matessi, 1983; Nowak et al., 2010; smith et al., 2010). Therefore, a more general inequality is required for which we will use Price's equation to derive (see Appendix A for full derivation or Queller, 1992). Similar to how we regress fitness, W, against genotype, G, in Price's equation, we will first regress fitness against an individual's genotype, G_X, and the genotype of its interactants, G_Y, which results in

$$W = W_0 + \beta_{WG_X}G_X + \beta_{WG_Y}G_Y + \varepsilon \tag{5}$$

where W_0 is an individual's base fitness and the β's are the partial regression coefficients. β_{WG_X} is the average effect an individual's, genotype has on its own fitness, ignoring the effect of the environment. For example, if the only difference between a cooperator, $G_X=1$, and a defector, $G_X=0$, is that a cooperator pays a cost, c, for others to benefit, then β_{WG_X} would be $-c$. Similarly, β_{WG_Y} is the average fitness effect the environment, G_Y, has on an individual's fitness, ignoring the effect of the individual's genotype. Again, when G_Y measures the number of cooperators in an individual's environment, and each cooperator gives its interactant a benefit, b, then β_{WG_Y} is b. The last term, ε, is the residual, which may be different for every individual and describes the difference between an individual's actual fitness and the fitness predicted by the regression. After substitution into (2) and simplifying, we get that a cooperative act is favored when

$$\beta_{G_YG_X}\beta_{WG_Y} + \beta_{WG_X} > 0 \tag{6}$$

where β_{WG_Y} and β_{WG_X} are the same as above and now $\beta_{G_YG_X}$ is a measure of how an individual's social environment covaries with the individual's genotype and is the linear regression definition of relatedness, r (Fig. 4). Thus, in the simplest case where every cooperator pays a fixed cost c for other individuals to gain a fixed benefit b – all measured in number of offspring – cooperators are favored when $rb-c>0$ (Fig. 5A).

Before going on to more complicated cases, a more thorough discussion of "relatedness" is warranted. Contrary to the popular use of the word, "relatedness" describes a population of interacting individuals, where r refers to how assorted similar individuals are in the population. r is 1 if every individual only interacts with genetically identical individuals ($G_X=G_Y$) and r is 0 if interactions are random (G_Y does not change with G_X). Thus, if we have ten clonal colonies, all from different strains of bacteria, and we put each one in a separate test tube to grow, this would be full assortment and r would be 1. Any otherwise dominated cooperative strains with $b>c>0$ would end up growing better than the defector strains and thus would increase in frequency in the ten test tube population because $1\cdot b-c>0$. On the other hand, if we take an equal number of cells from each colony and put a little bit in each of the ten test tubes, then there would be no assortment and r would be 0. Similarly, when two strains are competing in a well-mixed environment like a single test tube, then relatedness is again 0 because each individual is no more likely than average

to interact with its own type. In both these cases where $r=0$, any cooperators dominated by defectors would decrease in frequency, again consistent with Hamilton's rule because $0 \cdot b - c < 0$.

There is, however, a measure of how related two individuals in a population are. Importantly, Hamilton's rule asks if a trait will increase in *frequency* and not necessarily number, so even if we are only interested in the relatedness between two individuals, we still have to take into account the population. The inclusive fitness definition of relatedness describes how similar an actor of an action is to its recipient, relative to the population, and is given by

$$r = \frac{\mathbb{P}_{A\to R} - \mathbb{P}_{A\to P}}{\mathbb{P}_{A\to A} - \mathbb{P}_{A\to P}} \tag{7}$$

where $\mathbb{P}_{A\to R}$ is, formally, the probability that a gene drawn at random from the actor at the focal loci (e.g. the gene coding for the cooperative behavior) is identical in state to a gene drawn at random from the recipient at the focal loci (West et al., 2006). Similarly, $\mathbb{P}_{A\to P}$ compares the actor to a member of the population drawn at random and $\mathbb{P}_{A\to A}$ compares the actor to itself ($\mathbb{P}_{A\to A}=1$ for haploids). In the case of a rare gene in a large population, $\mathbb{P}_{A\to P}\to 0$, and $r\to \mathbb{P}_{A\to R}/\mathbb{P}_{A\to A}$ which gives the classic result that r is one half for siblings and one eighth for cousins (in the absence of inbreeding). Eq. (7) also gives $r=1$ for clonemates, and $r=0$ for two individuals drawn at random from the population. Note that the population "relatedness" is the average relatedness of interacting individuals, but for the purposes of this paper, we will stick to the linear regression definition of relatedness in Eq. (6) because it is easier to visualize (Fig. 4). For other definitions and methods of conceptualizing relatedness and assortment, see the supplemental text.

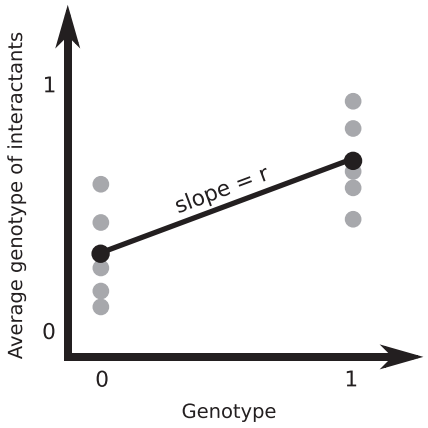

Fig. 4. Relatedness as a regression coefficient. To apply the general form of Hamilton's rule, we need to assign a number to each genotype, and if we are working with two strains (e.g. a cooperator and defector) we can arbitrarily set the cooperator genotype to 1 and the defector genotype to 0. The relatedness, r, is then the linear regression coefficient connecting an individual's genotype with the genotype of its interactants. Note that when everyone interacts with the same number of individuals, the regression passes through the average of each's interactants (black dots). This allows us to redefine relatedness as $r=\mathbb{P}(C|C)-\mathbb{P}(C|D)$: the probability for a cooperator to interact with a cooperator minus the probability for a defector to interact with a cooperator. Also note that scaling the x axis would also scale the y axis by the same amount, leaving the slope the same, so arbitrarily setting the genotypes to 0 and 1 does not lose generality.

Returning to our linear regression formula for Hamilton's rule (6), it is important to understand how this is applied to more complicated (i.e. realistic) situations. The fitness of a microbe will very rarely be a linear function of its environment, if only because linearity is just one of the infinite number of possibilities. When an individual's fitness is a non-linear function of its genotype and environment, then the linear regression definitions of the "benefits" and "costs" depend on the data points sampled in the population (Fig. 5B and C, data points are dark dots). Now the measured b and c are no longer inherent properties of the interaction, but rather functions of the population structure. In fact, b and c are completely dependent on the sampled data points and change every time they are measured.

The measured b and c can also give nonsensical results depending upon the population structure being sampled. For example, Hamilton's rule can predict negative fitness values for some cooperator frequencies (Fig. 5B). Alternatively, the measured b could be less than c for some population structures, which would incorrectly suggest that cooperation is in general impossible to evolve (Fig. 5C). Also, if at some point b is measured to be 2 and c to be 1, then as long as the relatedness, r, is greater than $c/b=\frac{1}{2}$ at that moment, cooperation will increase in frequency; however, this does not mean that $r>\frac{1}{2}$ is the general condition for cooperation to be favored because b and c change with the population structure and thus also with r. Note that while a linear regression for the costs and benefits can always be done, it often is uninformative. Rather than being a powerful predictive tool, this method can become a cumbersome way to add up fitnesses.

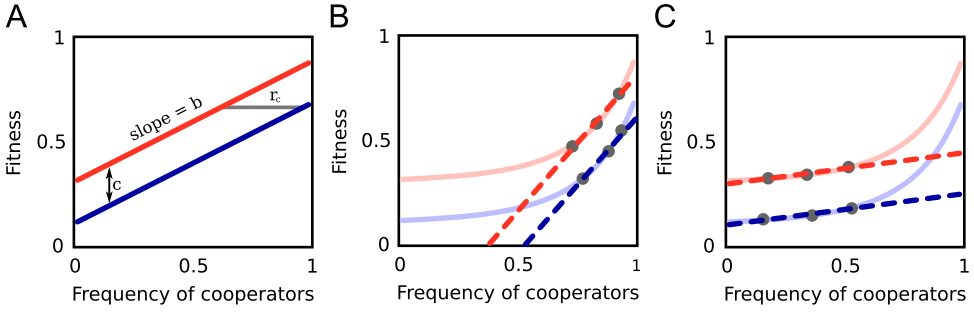

Fig. 5. Hamilton's rule is a linear regression to generally non-linear data. (A) In the simplest case, where everything is linear, the slope of the fitness function is b and the difference between a defector and cooperator's fitness with the same frequency of cooperator interactants is c. For a cooperator to have a higher fitness than a defector, it must interact with r more cooperators than defectors do (see figure). Because of the geometry, this results in a critical relatedness, $r_c=c/b$, above which cooperators are favored (i.e. when $rb-c>0$). Unfortunately, life, and particularly microbes, is rarely this simple. (B and C) When the fitness is a non-linear function of the fraction of cooperators (light lines), b and c become linear regression coefficients based on the sample points (gray dots). Unfortunately this masks many of the interesting (non-linear) qualities of the system and provides no prediction power because b and c change with the population structure. Also note that this method can lead to negative fitness values (B) and situations where $b<c$ (C), which would incorrectly suggest that cooperation can never evolve. Note that b and c determine the necessary r for cooperation to evolve, but b and c are in turn affected by the population structure and thus r, which makes disentangling fitness effects from population structure often impossible. It is important to note that the cooperator and defector fitnesses are not independently regressed, rather only one regression is done with the slope (b), vertical difference between defectors and cooperators at a fixed cooperator fraction (c), and base fitness (y-intercept) as the three parameters (adapted from smith et al. (2010)).

Also, on the practical side, G_Y is often hard to define. In the simplest case, where every individual interacts equally with the same number of individuals (e.g. in well-mixed test tubes seeded with the same number of cells), then G_Y is just the average genotype of the interactants. However, when different interactions are stronger than others, like on an agar plate where interactions weaken with distance, or when individuals interact with different numbers of individuals, then G_Y becomes more complicated (Le Gac and Doebeli, 2010). While Hamilton's rule can still be applied, the relatedness and benefit terms, $\beta_{G_Y G_X}$ and β_{WG_Y}, become less intuitive and potentially arbitrary. It should also be noted that when multiple alleles are competing, G_X, and thus also $\beta_{G_Y G_X}$, can become arbitrary. In fact, $\Delta\overline{G}$ loses much of its meaning in this case. Therefore, in cases of multiple competing alleles or nontrivial population structure, Hamilton's rule is often replaced with explicit modeling (e.g. see Xavier and Foster, 2007).

More complicated forms of Hamilton's rule are also possible. For example, one can take a linear regression of cooperator and defector fitnesses separately, resulting in two different slopes (Queller, 1985, see supplemental text for details). Importantly, this method can predict bistability and coexistence, a distinction that is traditionally out of reach for Hamilton's rule. While this can be a more accurate view of the interaction and should be favored in certain cases, it still cannot capture the full picture and has some of the same pitfalls of traditional Hamilton's rule without the advantages that come from its simplicity.

Alternatively, a highly modified form of Hamilton's rule has recently been suggested for the study of microbes (smith et al., 2010). To apply this unique formula, one first fits two (non-linear) regressions to the cooperator and defector fitnesses. A Taylor series expansion is then taken of these two functions and the coefficients of these Taylor series are used in vector forms of b and a new synergy term d. r is now a vector containing the traditional definition of relatedness, but also additional terms describing higher order moments of assortment (Godfrey-Smith and Kerr, 2009). In this formulation, the population structure is completely separated from the fitness effects—that is, b, c, and the synergy term d are now constants and do not change with each iteration of the calculation. We see smith et al.'s reformulation as a major step forward for the application of Hamilton's rule to microbes, but it is not without its limitations. First, although it may be a more accurate view of microbial interactions, higher order relatedness and fitness terms can be much less intuitive than Hamilton's simple rule. Also, it cannot accommodate multiple strains competing or dependence on density or time, at least as it is currently written. Finally, the reformulation still is not a replacement for a mechanistic model, which can identify biologically relevant parameters and aid in predictions.

5. Weak selection

Before moving on to alternative methods of conceptualizing and analyzing cooperation, let us discuss why the simple form of Hamilton's rule – without any regressions – is usually inappropriate for the study of microbes. Hamilton thought of evolution as a very slow and gradual process where each mutant varied only slightly from the wild type and this can be seen in many of the assumptions used in deriving the simple form of Hamilton's rule, namely the assumption of weak selection (Hamilton, 1964; Wenseleers, 2006). In the kin selection literature, (δ) weak selection refers to the assumption that everyone in the population plays approximately the same strategy and any mutant can only play a slightly different strategy (wild type strategy $\pm\delta$ as $\delta\to 0$; Wild and Traulsen, 2007). This is analogous to every microbe in a population producing the same amount of a public good like invertase and the only mutations allowed are those that produce slightly more or slightly less invertase. Any mutants, therefore, also only have a vanishingly small difference in fitness, leading to dynamics dominated by random drift.

In evolutionary game theory, a similar concept called w weak selection is often used (Nowak et al., 2004). In contrast to δ weak selection, individuals in the population can play distinct, discrete strategies, but the fitness difference between any two strategies is always very small (mixed strategies are also allowed depending on the model). This would correspond to two yeast cells that produce the maximum and minimum amount of invertase having only an infinitesimal difference in fitness. While weak selection is often useful for gaining insight by simplifying the dynamics in analytical models, it is not the general case and is often invalid for cooperative microbial behaviors because of large differences in fitness.

The intuition behind weak selection partly comes from the idea that any one interaction between two animals often has only a small effect on their fitness; for example, whether or not an animal shares a berry with its group on a particular day is unlikely to have a large impact on anyone's survival. However, many forms of microbial interactions are literally do or die. If sucrose is the only available sugar, then a microbe will starve if it cannot break it down. The difference in generation times is also important because while an animal may have many small interactions in its much longer lifetime, a microbe is likely to have only a few interactions, many of which will be important in determining if it will divide in the next hour. Additionally, while the complexity of animal behavior makes evolution by small changes more likely in animals, mutations of large effect are common in microbes.

So the concept of weak selection is inappropriate for microbes, why is this important? First of all, while the linear regression form of Hamilton's rule still works, inclusive fitness theory and many of its results are provably correct only in the limit of weak selection (Nowak et al., 2010). For example, the commonly cited rule that natural selection acts to maximize inclusive fitness has only been proven using stringent assumptions, including weak selection (Grafen, 2006; van Veelen, 2007). Also, assuming weak selection gives a quantitatively different answer than more accurate models (Queller, 1984; Wenseleers, 2006, see supplemental text for details). This is particularly relevant when empiricists apply results from a weak selection model to actual data. In addition, when empiricists cannot measure the cooperative allele of interest, they often use neutral genetic markers as a proxy to estimate relatedness. Unfortunately, measuring relatedness this way is only valid when the markers and the allele of interest are under weak selection (Queller and Goodnight, 1989; Hardy, 2003). Because genes like invertase and other cooperative alleles are often not under weak selection, using a genetic marker approach to measure relatedness in microbes will likely give a wrong result. In fact, despite their seemingly simpler genetics, relatedness is harder to estimate for microbes than for animals (West et al., 2006).

Also, even if the costs and benefits of an interaction are linear functions of one's genotype and environment, the measured b and c will not be linear unless selection is weak (Ross-Gillespie et al., 2007). This results from growth being an exponential process, so the total growth difference between a cooperator and defector grows exponentially with the costs and benefits of the interaction. This will be a linear function only when b and $c \ll 1$, which is the limit of weak selection (see supplemental text for details). Another way to arrive at the same conclusion is by imagining that, in the limit of δ weak selection, every individual's phenotype would be almost the same, so all the gray dots in Fig. 5B and C would be right next to each other. This closeness allows the linear regressions to be replaced by derivatives,

turning the otherwise hard frequency-dependent situation into an easy frequency-independent maximization problem (Frank, 1998; Wenseleers et al., 2009). While this may be alluring to the theorist, this technique is often ill-suited for the empirical study of microbial cooperation.

6. Multilevel selection

Multilevel selection, also known as group selection, has gone through its ups and downs, from once being completely discredited to now being acknowledged as an important organizing principle that can aid in the evolution of cooperation (Traulsen and Nowak, 2006; Nowak, 2006b). Multilevel selection occurs whenever selection acts on multiple levels, such as when individuals are partitioned into groups and the groups themselves compete – either directly or indirectly – in addition to the individuals inside each group competing against themselves. In this case, traits that are costly to the individual but beneficial to the group can be selected for if the between-group selection is stronger than the within-group selection. However, without any mathematical modeling, this statement is empty and is even dangerous because it can easily be used to justify any altruistic behavior. Thus, let us quickly go through the derivation of a simple multilevel selection model to better understand the math behind selection at two levels. We start by assuming a population is made up of several segregated groups, each with a total fitness, W_g, and genotype G_g. Now we use the full version of Price's equation:

$$\overline{W}\Delta\overline{G} = \mathrm{Cov}(W_g, G_g) + \overline{W_g \Delta G_g}. \tag{8}$$

However, unlike before, $\overline{W_g \Delta G_g}$ cannot be disregarded because the average genotype of the group changes, while before we only assumed that individual genotypes stayed the same. Fortunately, $\overline{W_g \Delta G_g}$ is similar to the left side of the equation and another iteration of Price's equation is possible:

$$\overline{W}\Delta\overline{G} = \mathrm{Cov}(W_g, G_g) + \overline{\mathrm{Cov}_g(W_i, G_i)} + \overline{W_i \Delta G_i} \tag{9}$$

$$\overline{W}\Delta\overline{G} = \mathrm{Cov}(W_g, G_g) + \overline{\mathrm{Cov}_g(W_i, G_i)} \tag{10}$$

where $\overline{W_i \Delta G_i}$ is now disregarded because i is the individual level here. If, however, we wanted to model more than two levels, we could just keep iterating Price's equation. Now $\mathrm{Cov}(W_g, G_g)$, the relationship between group fitness and group genotype, represents the between-group selective force and $\overline{\mathrm{Cov}_g(W_i, G_i)}$ is the average within-group selective disadvantage of being a cooperator and will likely be negative. If the between-group selection advantage to being a cooperator is greater than the within-group disadvantage, then $\Delta\overline{G} > 0$ and cooperation spreads.

Note that because $\mathrm{Cov}(W_g, G_g) = \beta_{W_g G_g} \mathrm{Var}(G_g)$ (by the definition of covariance), the between-group selection pressure is proportional to the variance between groups. In fact, connecting this with the idea of assortment, $\mathrm{Var}(G_g)$ is maximized when each group is either all cooperators or all defectors ($G_g = 1$ or 0 for each group), which would correspond to full assortment of genotypes and an r of 1. Similarly, when every group has the same composition of genotypes, $\mathrm{Var}(G_g) = 0$, and there is no assortment of genotypes, $r = 0$.

These connections should come as no surprise because when the population structure is simple and everything is linear, assortment is *the* fundamental explanation for the evolution of altruism (Fletcher and Doebeli, 2009). How this assortment happens in nature, and not necessarily how one measures it, then becomes the most important question (Nowak, 2006b; Taylor and Nowak, 2007). One possible mechanism is active kin discrimination, where individuals show a preference towards relatives when segregating into interaction groups. This has been shown to be a major mechanism for the maintenance of cooperative spore formation in the social amoeba *Dictyostelium purpureum* (Mehdiabadi et al., 2006). Also, spatial structure such as the simple partitioning into groups can favor cooperation because whenever smaller subpopulations are initiated by a random sample of M number of cells, the level of assortment in the total population is $1/M$, which can be significant for small M (Fletcher and Zwick, 2004; Ackermann et al., 2008; Chuang et al., 2009). The assortment is even higher if there is additional spatial structure in each subpopulation such that each cell line interacts more often with itself than others in the subpopulation. Care should be taken with this argument, however, because this may also mean that each cell line competes more intensely with itself for resources and reproduction, potentially canceling out any benefit of assortment (Queller, 1994). This is why simple population structure or global competition is often assumed beforehand and is part of why relatedness measurements alone are inconclusive.

To contrast multilevel selection and kin selection, emergent properties are often better understood using multilevel selection math and arguments than by Hamilton's rule. By emergent properties we mean any property of the system that cannot be easily described as a sum of smaller effects; standard examples of emergent properties include multicellularity and collective behavior where groups themselves often compete directly. Because emergent properties are more than just a sum of their parts, they are often characterized by non-linearities, which is perhaps the main reason why Hamilton's rule is less predictive in these situations.

By applying the same model to every situation, Hamilton's rule and multilevel selection theory can mask many of the important qualities of a system. True understanding comes only from explicit modeling and not from simply examining statistical quantities, which the Price equation, and any rule derived from it, coaxes us all to do. This point is best made by examining Anscombe's quartet, four datasets with identical summary statistics, but strikingly different graphs (Fig. 6; Anscombe, 1973). Each of these datasets should be modeled and understood in distinct ways; too much information is lost when we resort to plugging numbers into covariances without conceptualizing the underlying data-generating process. Note how ill-suited a linear regression is for some of these datasets; this same situation happens when Hamilton's rule is applied to potentially non-linear microbial data.

7. Common misconceptions

Perhaps the biggest misconception in the study of the evolution of cooperation is that there is something special, or even magical, about kin selection that allows it to explain phenomena that other theories cannot. Inclusive fitness, the basis of kin selection, is the effect of an individual's action on everyone's fitness weighted by the relatedness between the individuals; this is simply an alternative accounting scheme and, therefore, cannot produce novel predictions outside of the scope of standard natural selection theory (Wenseleers et al., 2009; Nowak et al., 2010). It is also under contention whether or not inclusive fitness is even defined if certain assumptions are not met, such as: weak selection, additivity, and simple population structure (Grafen, 2006). Inclusive fitness also cannot describe dynamics, which immediately makes it less general than standard techniques like evolutionary game theory (Traulsen, 2010). It should also be mentioned that evolutionary game theory has become an extremely general and powerful tool for understanding evolutionary dynamics (Nowak, 2006a). In particular, game theory is not restricted to the use of game matrices which are often

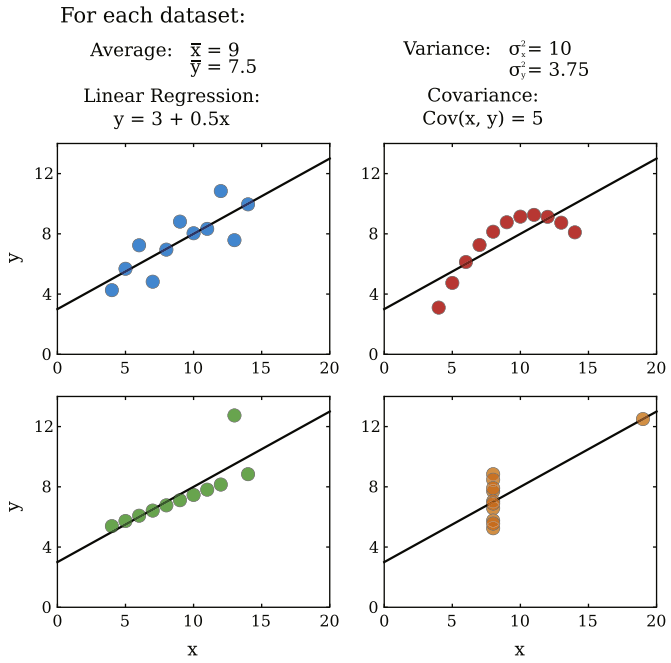

Fig. 6. Anscombe's famous quartet illustrates the need of visualizing one's data before statistically analyzing it. All four datasets have identical summary statistics (e.g., mean, variance, regression coefficients, and covariance), but vastly different graphs, which are more important in interpreting the data. Researchers should graph and interpret their data before blindly applying Price's equation or Hamilton's rule without a model. Importantly, if x was the genotype and y the fitness, Price's equation would predict the same change in average genotype in all four populations, but we would still know nothing about the underlying dynamics if we fail to visualize the data and model the situation. (adapted from Anscombe (1973)).

inappropriate for interactions that are not pairwise or linear (Frey, 2010).

Simply measuring the personal fitness of each individual is sufficient to explain altruism, without directly taking relatedness into account because a gene coding for an altruistic behavior will only spread if its bearers have a higher fitness on average. This point is often misconstrued, especially when considering inclusive fitness; for example, Hamilton mentions in his original 1964 paper that "a gene may receive positive selection even though disadvantageous to its bearers if it causes them to confer sufficiently large advantages on relatives." Of course a gene cannot spread if the end effect is disadvantageous to its bearers; the bearers have to also on average receive a sufficiently large advantage from others to outweigh the costs. The problem with this quote, and other common informal inclusive fitness arguments, is the creation of a false dichotomy between "bearers" and "relatives," when in reality the only relatives that matter are those that are also "bearers" (Fletcher and Doebeli, 2009).

There is also the misconception that inclusive fitness calculations are generally easier than other approaches. While there are cases under certain assumptions where this has been found to be true (Taylor et al., 2007), inclusive fitness measurements need all of the same information as normal techniques and are often more indirect and easier to mess up. In fact, even experts are not immune to errors when applying Hamilton's rule; for example, Kümmerli et al. (2010) use the simple form of Hamilton's rule to predict changes in cooperator frequency in an experimental bacterial system with non-linearities. Unfortunately, because the data did not fit a linear model, the more complicated linear regression or Queller's synergy reformulation was necessary (see supplementary text for details). In a different paper, two of the same authors admit that a "naïve application of Hamilton's rule may lead to mistakes. For this reason, it is easier to use standard population genetics, game theory, or other methodologies to derive a condition for when the social trait of interest is favored by selection" but go on to say that Hamilton's rule should be used "as an aid for conceptualizing this result." While we agree that using as many perspectives as possible is often the best way to understand a situation, and that Hamilton's rule can be a useful intuitive tool if used correctly, the additive nature implicitly assumed by Hamilton's rule often makes it inappropriate for conceptualizing non-linear microbial models. Also, because it requires transformations of variables, the end result in the form of Hamilton's rule is often visually unconnected from the original mechanistic model, making it difficult to judge how changes in the original model will affect the transformed inequality. Note that Hamilton's rule has been used correctly in the study of microbes (e.g. see Gilbert et al., 2007), but the chance for error is high, especially if certain assumptions do not apply like a simple population structure (note also that the cited paper assumed weak selection when measuring relatedness, which is likely to give an overestimate).

A game theoretical viewpoint can be crucial and an important insight could be nearly impossible to see without it. For example, in a recent theoretical paper, we used evolutionary game theory to study, both analytically and via simulations, host-symbiont interactions (Damore and Gore, 2011). We showed that in a generic population structure, when a symbiont evolves much faster than its host – which is often the case – the equilibrium distribution of strategies played by the host and its symbionts is the same as the sequential game equilibrium where the host moves first and the symbiont second. This fundamental insight inspired by game theory applies to parasites and mutualists alike, explaining both the Red Queen hypothesis and the apparent enslavement of endosymbionts.

Additionally, confusion about the definition of relatedness is common among empiricists. Because Hamilton's rule is concerned about an increase in frequency of a gene and not necessarily an overall increase in number, every definition of relatedness must take into account the population. Therefore, relatedness is not the percent of genome shared, genetic distance, or any extent of similarity between two isolated individuals in a larger population. Also, because horizontal gene transfer is commonplace between microbes and selection is strong, phylogenetic distance or any other indirect genetic measure is likely to be inaccurate. Many of these false definitions live on partly because ambiguous heuristics like "$\frac{1}{2}$ for brothers, $\frac{1}{8}$ for cousins," which require very specific assumptions, are repeated in the primary literature. Also, most non-theoretical papers simply define relatedness as "a measure of genetic similarity" and do not elaborate or instead leave the precise definition to the supplemental information (West et al., 2006). Unfortunately, scientists can easily misinterpret this "measure of genetic similarity" to be anything that is empirically convenient such as genetic distance or percent of genome shared. Largely because of this confusion, we support the more widespread use of the term "assortment," which is harder to misinterpret (Ackermann et al., 2008). For similar reasons of reader understanding, we also encourage authors to make calculations more explicit, either in the main or supplemental text, and to avoid repeating previous results without giving the assumptions that went into deriving them.

There is also an overemphasis on genotypic relatedness, which is not strictly required for the evolution of altruism. To illustrate our point, we will repeat an example from Fletcher and Doebeli (2009), which we found particularly illuminating. Consider a haploid microbial species that can produce a public good coded by two completely different mechanisms, each encoded at a different locus. At the first locus, allele A produces the public good, while allele a does not. Similarly, at the second locus, allele B produces the public

good and allele *b* does not. Now if *Ab* individuals only interact with *aB* individuals and *ab* individuals interact with other *ab* individuals, cooperation will spread, but it is not because of a genotypic similarity between individuals that interact. Cooperation evolves because individuals with an altruistic genotype are positively assorted with other individuals that have cooperative *phenotypes* (see also Appendix A). This may seem to be somewhat of a semantic issue, but this distinction is ultimately important for evaluating different models and deciding the necessary requirements for the evolution of cooperation. Mutualisms between species – where the concept of "relatedness" is clearly out of place – also exemplify the usefulness of the term "assortment" (Foster and Wenseleers, 2006). Nevertheless, even assortment is unnecessary when there are non-linear effects (Hauert et al., 2006).

8. Discussion

For researchers studying microbial interactions, the most important steps towards understanding the system are experimental measurements and modeling (Brännström and Dieckmann, 2005; MacLean and Gudelj, 2006; Xavier and Foster, 2007). Without even a rough model of the interaction, no predictions or explanations of phenomena can be made. Unfortunately, the simplicity of Hamilton's rule often discourages researchers from doing any modeling beyond the simple idea that cooperators pay a fixed cost, *c*, for others to gain a fixed benefit, *b*. Importantly, mechanistic models are also often required to guide the researcher to measure the relevant biological parameters. While simplicity can aid initial comprehension, it is exactly the details and non-linearities that give rise to many of the interesting qualities in microbes like graded responses, collective behavior, and coexistence between multiple strains and species (West et al., 2006; Mehta and Gregor, 2010; Reichenbach et al., 2007; Gore et al., 2009).

On a similar note, researchers are often too quick to conclude that a behavior can be explained by kin or group selection without specifying any of the details or how assortment might happen in nature. Identifying a behavior as cooperative is just the beginning; in fact, many techniques cannot even distinguish all four types of interactions mentioned in Section and Fig. 1, which we see as a fundamental first step in understanding the interaction. For example, observing that pure cooperator cultures grow faster than pure defector cultures tells you nothing except how to label the strains because the interaction could still be any of the four basic types (or even a combination of these four types). This is a major problem because these situations should be studied and thought of in distinct ways. Unfortunately, social interactions are often classified as one of four types: altruistic, mutually beneficial, selfish, or spiteful; these four categories simply correspond to the sign of *b* and *c* and cannot allow for coexistence or bisability because more than two numbers are required to describe them.

Authors should also be more explicit about what they mean when they say "fitness." While we have only used "fitness" to mean the total number of offspring here because that is what is used in the Price equation and Hamilton's rule, "fitness" has also been used to mean growth rate which is often more intuitive to model and conceptualize (Kerr et al., 2002, 2006; MacLean and Gudelj, 2006; Gore et al., 2009). In particular, growth rate is the relevant quantity when using differential equations, a powerful modeling tool. We support both uses, as long as the authors are especially clear because semantic differences are common between game theory and kin selection, which has created confusion in the past (see supplemental text for details). With this said, no matter what definition of fitness one uses, absolute fitness is often more informative than relative fitness because absolute fitness is the quantity used in calculations. Also, even if the relative fitness profile is the same for two populations they could still have drastically different potentials for cooperation and similarly even if the relative fitness profiles are significantly different, they have the same potential for the evolution of cooperation (see supplemental text for details).

Biology thrives on the study of model organisms. Model systems in microbial cooperation include: slime mold and bacterial fruiting bodies, biofilm formation, quorum sensing systems, sucrose metabolism, restrained fermentation, bacteriocin and siderophore production, persistence, programmed cell death, virulence, cooperative antibiotic resistance, swarming, and many others (Strassmann et al., 2000; smith et al., 2010; Xavier and Foster, 2007; Diggle et al., 2007; Gore et al., 2009; MacLean and Gudelj, 2006; Kerr et al., 2002; Kümmerli et al., 2010; Lewis, 2006; Kerr et al., 2006; Turner and Chao, 1999; Dugatkin et al., 2005; Chuang et al., 2010; Lee et al., 2010; Velicer and Yuen-tsu, 2003). In fact, collective behavior is beginning to be seen as the norm in microbes, rather than the exception (for reviews of social behavior and cooperation in microbes see Crespi, 2001; Velicer, 2003; Travisano and Velicer, 2004; West et al., 2006, 2007; Brown and Buckling, 2008; Frey, 2010; Hibbing et al., 2010; Mehta and Gregor, 2010). However, the necessary first steps for studying a cooperative behavior, explicit modeling and measurement of biologically relevant parameters, have yet to be done for many of these systems, a fruitful avenue for future research. Also, each discovery of a new cooperative system advances our knowledge of how cooperation evolves and is maintained in nature.

Because science is fruitless if it lacks questions to answer, here we summarize some of the many important questions in the field of microbial cooperation. First, what produces the necessary assortment and non-linearities for cooperation to evolve in natural and experimental microbial systems? Similarly, what effects and characteristics are lost in the lab? Many features that may favor cooperation, like clumping, are selected against by standard lab techniques and are, therefore, missing in some domesticated strains (Aguilar et al., 2007). What is the speed and strength of selection in natural systems? How do green beard genes evolve? How relevant is horizontal gene transfer to the maintenance and spread of cooperation (Nogueira et al., 2009)? How are interspecific mutualisms formed and maintained (Doebeli and Knowlton, 1998)? What is the correct spatial scale for microbial interactions (Whitaker, 2009)? And, finally, how can these results be applied to fight disease and promote cooperation in synthetic systems (Axelrod et al., 2006; Wintermute and Silver, 2010)?

We applaud Hamilton for pointing out the important insight that genes "care" about not only themselves, but also about identical copies in other organisms. If well understood, his crucial observation can be a powerful intuitive tool, a first step to understanding how or why a behavior might evolve. Unfortunately, the limitations and indirect nature of inclusive fitness make it nearly impossible to correctly apply quantitatively and, similar to group selection, can lead to false intuitions if misused. Rather than resorting to very general forms of Hamilton's rule and multilevel selection that often lack specificity and prediction power, we conclude by advocating for experimentally inspired and tested models to better understand the evolution of microbial cooperation.

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Appendix A. Derivation of Hamilton's rule

We will derive Hamilton's rule similarly to Queller (1992). First, let us perform a linear regression on the fitness of an organism, W, with respect to the individual's genotype, G_X, and the average genotype of its interactants, G_Y, as independent variables. The fitness of any one organism is then

$$W = W_0 + \beta_{WG_X} G_X + \beta_{WG_Y} G_Y + \varepsilon \tag{A.1}$$

where W_0 is the organism's base fitness and the β's are partial regression coefficients. The last term, ε, is the residual, which may be different for every individual and describes the difference between an individual's actual fitness and the fitness predicted by the regression. Substitution into (2) gives

$$\overline{W}\Delta\overline{G} = \mathrm{Cov}(W_0,G_X) + \beta_{WG_X}\mathrm{Cov}(G_X,G_X) + \beta_{WG_Y}\mathrm{Cov}(G_Y,G_X) + \mathrm{Cov}(\varepsilon,G_X) \tag{A.2}$$

The β's drop out of the covariances because they are constants. Similarly, $\mathrm{Cov}(W_0,G_X)$ is zero because W_0 is a constant. Importantly, $\mathrm{Cov}(\varepsilon,G_X)$ is also zero because the residuals are necessarily uncorrelated with the independent variables of a linear regression. Now, because $\overline{W}$ is positive, the necessary condition for $\overline{G}$ to increase is

$$\overline{W}\Delta\overline{G} = \beta_{WG_X}\mathrm{Cov}(G_X,G_X) + \beta_{WG_Y}\mathrm{Cov}(G_Y,G_X) > 0 \tag{A.3}$$

Now, because $\mathrm{Cov}(G_X,G_X) = \mathrm{Var}(G_X)$ is necessarily non-negative, we can divide both sides by $\mathrm{Var}(G_X)$ and maintain the sign of the inequality:

$$\beta_{WG_X} + \beta_{WG_Y}\frac{\mathrm{Cov}(G_Y,G_X)}{\mathrm{Var}(G_X)} = \beta_{WG_X} + \beta_{WG_Y}\beta_{G_YG_X} > 0 \tag{A.4}$$

which is the equation used in the main text. We should also mention that we personally prefer the formula

$$\beta_{WG_X} + \beta_{WP_Y}\frac{\mathrm{Cov}(P_Y,G_X)}{\mathrm{Var}(G_X)} > 0 \tag{A.5}$$

where G_Y is replaced with P_Y. This emphasizes the important point that altruistic genes spread when they are assorted with the altruistic phenotypes of others. The original form is presented in the text because it is more widely used and simpler.

Appendix B. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtbi.2011.03.008.

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