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Original Article

Spontaneous nongenetic variation of group size creates cheater-free groups of social microbes

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In social organisms, cheaters that gain a fitness advantage by defecting from the costs of cooperation reduce the average level of cooperation in a population. Such cheating load can be severe enough to cause local extinction events when cooperation is necessary for survival, but can also mediate group-level selection against cheaters across spatially structured groups that vary in cheater frequency. In cheater-laden populations, such variation could be generated by the formation of new homogeneous groups by small numbers of identical cells. Here, we use the model social bacterium *Myxococcus xanthus* to test whether population bottlenecks inherent to the starvation-induced formation of multicellular fruiting bodies can generate cheater-free groups within an initially cheater-laden population. We first show that genetically identical fruiting bodies vary greatly in their numbers of stress-resistant spores. We further show mathematically and experimentally that this variation can include small cheater-free groups. Such nongenetic variation in group size was found to occur in a variety of *M. xanthus* isolates and *Myxococcus* species. Our results suggest that stress-induced reductions in group size may serve as a general process that repeatedly purges genetic diversity from a minority of social groups, thus recurrently generating high-relatedness social environments unburdened by cheating load.

Key words: bet hedging, bottleneck, multicellular development, natural variation, phenotypic heterogeneity.

INTRODUCTION

Many factors influence the average degree of cooperation that evolves at any given cooperative trait in any given population. Within the framework of kin-selection theory (Michod 1982; West et al. 2007; Frank 2013), these factors can be most broadly categorized as those that influence the average degree of relatedness among interactants (Griffin and West 2003; Foster et al. 2004; Kuzdzal-Fick et al. 2011) and those that influence the relative costs and benefits of expressing cooperative traits (Gore et al. 2009; Waibel et al. 2011; Mitri and Foster 2016). Within the framework of multi-level selection theory (Wilson 1975; Goodnight 2013; Simon et al. 2013), the determinant factors are formulated as the relative degree of selection within versus across social groups (Traulsen and Nowak 2006; Wilson and Wilson 2007). In both frameworks, in order for alleles that promote individually costly acts of cooperation to persist, the benefits of cooperation must be preferentially exchanged among

cooperators, i.e. individuals that express a cooperative trait, relative to exchanges from cooperators to noncooperators (Le Gac and Doebeli 2010).

The average degree of relatedness between cooperators and recipients of cooperation in a population is influenced by counteracting forces that limit versus promote organismal dispersal (Lion and Baalen 2008; Kümmerli et al. 2009) and can also be affected by individual behaviors that cause cooperative actions to be preferentially directed toward kin, even when close kin and non-kin are spatially interspersed, i.e. kin discrimination (Strassmann et al. 2011; Gilbert et al. 2012). In populations composed of discrete social groups, relatedness can be conceived in terms of the relative degree of variance at a focal social gene or trait within versus across groups (Goodnight 2013). In such populations, local bottlenecks can also affect within-group relatedness and thus have the potential to promote the maintenance of cooperation (Brockhurst 2007; Nadell et al. 2010; Kraemer and Velicer 2011). For example, migration by a single clonally reproducing individual or by small homogeneous groups to a new site can lead to the establishment of high-relatedness groups (Travisano and Velicer 2004; Kraemer and Velicer 2011). Analogously, many multicellular organisms undergo unicellular bottlenecks during

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reproduction and thereby eliminate within-organism genetic variation and concomitant potential for evolutionary conflict (Grosberg and Strathmann 1998; Ostrowski and Shaulsky 2009). Population bottlenecks caused by stress-induced death can also decrease within-group diversity and thus influence social evolution (Ostrowski and Shaulsky 2009). In one study, the degree to which cooperation was reduced by cheating load in laboratory populations of the bacterium *Pseudomonas aeruginosa* correlated with bottleneck population size (which was experimentally manipulated), presumably because populations that underwent smaller bottlenecks maintained higher relatedness (Brockhurst 2007).

Investigations of natural variation in microbial traits have largely focused on heritable variation because it is the core of adaptive evolution (Arber 2000). Yet, many microbial phenotypes also vary at the cellular level among genetically identical individuals, for instance natural competence or tolerance to antibiotics (Balaban et al. 2004; Avery 2006; Losick and Desplan 2008; Nikolic et al. 2013; Viney and Reece 2013). This phenomenon has been termed phenotypic heterogeneity (Elowitz et al. 2002; Ackermann 2015). However, because this label does not clearly distinguish between genetically versus nongenetically based phenotypic variation, we refer to this phenomenon here as “nongenetic phenotypic variation.” Some forms of nongenetic phenotypic variation among individual bacterial cells have been proposed to represent bet-hedging strategies (Veening et al. 2008; Olofsson et al. 2009), although whether any given form is adaptive is often questionable. Here, we highlight that nongenetic phenotypic variation also occurs naturally at the group level in some microbial species and propose that nongenetic variation in the size of spontaneously formed cooperative groups can influence the evolution of cooperation.

In the myxobacteria (Velicer and Vos 2009) and other microbes that form discrete social groups (Branda et al. 2001; Fortunato et al. 2003), group size may vary nongenetically (as might other group-level traits). *Myxococcus xanthus* is a soil-dwelling myxobacterium and a model organism for the evolution of cooperative traits. Upon shortage of amino acids, *M. xanthus* cells initiate a developmental process in which a complex signaling cascade induces cellular aggregation into high-density mounds (Kroos 2007; Zusman et al. 2007). Many authors over the past 3 decades have reiterated a common estimate that ca. 10^5 cells aggregate to form each fruiting body (Shimkets 1999; Kroos 2007), but the source of this estimate is unclear. Inside developing fruiting bodies, a minority of cells (often estimated between 1% and 10%) differentiate into metabolically quiescent spores that are resistant to heat and desiccation, whereas the majority remain as undifferentiated rods or lyse. Spores subsequently germinate once conditions become favorable for growth. Because nonspores die under sufficient stress, fruiting body development may often involve severe bottlenecks in the size of local *M. xanthus* groups (Fiegna and Velicer 2003).

Myxobacterial development is a cooperative trait and is, thus, subject to cheating (Dao et al. 2000; Velicer et al. 2000). Developmental cheaters in *M. xanthus* are often behaviorally obligate defectors that perform poorly as clonal groups (i.e. produce few or no spores) but outperform cooperators at sporulation within mixed groups (at least when cheaters are in the minority), increasing their short-term fitness presumably by defecting from the costs of expressing a cooperative trait (Velicer et al. 2000; Fiegna et al. 2006; Velicer and Vos 2009). However, cheaters greatly decrease group productivity when they reach high within-group frequencies (Velicer et al. 2000; Fiegna and Velicer 2003; Fiegna et al. 2006; Ross-Gillespie et al. 2007), such that cooperator cells in cheater-free

groups have a fitness advantage over both cooperators and cheaters in chimeric groups burdened by a high frequency of cheaters (Velicer and Vos 2009; Strassmann and Queller 2011). Thus, any mechanisms that can generate cheater-free groups within an initially cheater-laden population would promote cooperation relative to scenarios in which such cheater-free groups are not generated.

Although previous studies have shown that *M. xanthus* natural isolates collected at different spatial scales display substantial heritable natural variation in many quantifiable social phenotypes (Fiegna and Velicer 2005; Vos and Velicer 2008; Kraemer et al. 2010; Morgan et al. 2010), none have tested for nongenetic variation in the size of fruiting-body groups. Here, we first quantify the degree to which spatially discrete fruiting bodies vary in the number of viable stress-resistant spores they contain, both in a domesticated reference strain and among several natural isolates. We then test whether stress-induced population bottlenecks, such as those caused by starvation, have the potential to purge smaller social groups of cheater genotypes. Finally, we ask whether fruiting body group-size distributions and the number of fruiting bodies produced by a given number of starting cells vary heritably across genotypes and thus might potentially be subject to selection.

MATERIALS AND METHODS

Strains

Strains used in this study and their developmental sporulation efficiencies in pure culture are described in Supplementary Table S1. Strain GJV1 is a laboratory descendant of the reference strain DK1622 (Kaiser 1979) and is distinguished from it by 5 accumulated mutations of unknown effect (Velicer et al. 2006). GJV1* is a lab-evolved strain that descends from a rifampicin-resistant clone of GJV1. The evolution experiment has been previously described (Rendueles et al. 2015b; Rendueles and Velicer 2017). Briefly, a clone of *M. xanthus* was allowed to swarm in CTT (10 mM Tris pH 8.0, 8 mM MgSO_4 , 10 g/L casitone, 1 mM KPO_4 ; Kaiser 1979) plates containing 1.5% agar and every 2 weeks a population sample from the most distal point of each expanding swarm (or from a random location on the leading edge for circular swarms) was transferred to the center of a new plate. This process was repeated for 40 cycles. GJV1* corresponds to population P8 c40 in (Rendueles et al. 2015b). The cheater strain OC (aka GJV32) was first described in (Velicer et al. 2002) and the specific strain used here (OC_{rif}, aka GJV82) is a spontaneous rifampicin-resistant variant of OC (Manhes and Velicer 2011). GJV32 and GJV82 share the same developmental defect and cheating phenotype and we therefore refer to GJV82 here also as “OC.” OC differs from its ancestor by 14 SNPs (Goldman et al. 2006). The isolation locations of natural isolates are indicated in Supplementary Table S1.

Bacterial cultures

Bacteria were inoculated from frozen stock cultures onto CTT hard (1.5%) agar plates and incubated for 3 or 4 days at 32 °C, 90% rH. Prior to experiments, strains were grown in 8 mL CTT liquid for 24 h at 32 °C with constant shaking (300 rpm) until they reached mid-exponential phase ($\text{OD}_{600} = \sim 0.5\text{--}0.7$). Cell densities of exponential-phase cultures were estimated with a TECAN Genios™ plate reader using a previously established standard curve.

Developmental assays

To initiate all experiments, liquid cultures in mid-growth phase were centrifuged and resuspended in TPM starvation buffer (10 mM Tris

pH 8.0, 8 mM MgSO₄, 1 mM K₂PO₄) to a final density of $\sim 5 \times 10^9$ cells/mL. For each assay, 50 μ L of resuspended culture were inoculated in the center of either TPM starvation plates (1.5% agar) or CF plates (10 mM Tris pH 8.0, 1 mM KH₂PO₄, 8 mM MgSO₄, 0.02% (NH₄)₂SO₄, 0.2% citrate, 0.1% pyruvate, 150 mg/L casitone and 1.5% agar). Developmental plates were incubated for 5 days at 32 °C, 90% rH, after which the population phenotypes were photographed. Individual fruiting bodies were then picked randomly (unless indicated otherwise) with sterile wooden toothpicks, to which whole discrete and mature fruiting bodies easily attach. After being picked, no debris was observed at the locations from which fruiting bodies were picked, suggesting successful recovery of the vast majority of fruiting body-associated cells. To count the number of viable spores within individual fruiting bodies, each of them was transferred into 500 μ L of ddH₂O and heated at 50 °C for 2 h to kill nonspore cells. We report spore numbers of all fruiting bodies in which any spores were detected. In a few instances, we obtained zero spores from an attempted fruiting body harvest. These are not taken into account as these instances may represent unrecognized failures to successfully harvest a fruiting body rather than fruiting bodies with no spores. Plates used to count total viable spores from an entire population of fruiting bodies were inoculated simultaneously (i.e. from the same starting cultures) as those used to count number of viable spores within individual fruiting bodies. For the whole-population counts, all fruiting bodies were harvested with a scalpel blade, transferred into 1 mL ddH₂O, and heated at 50 °C for 2 h. All samples were then sonicated by microtip, diluted into CTT plates (0.5% agar), and incubated for 7 days before the colonies were counted. The number of fruiting bodies formed on each plate were counted using the cell counter included in ImageJ 1.42q software developed by the NIH (<http://imagej.nih.gov/ij/download.html>).

Model of spore survival

A theoretical model of cheater-cell number per fruiting body was constructed in R version 3.1.2 with the aid of lattice and lattice-Extra packages for plotting (Figure 2A). The absolute number of stress-resistant spores of a cheater genotype in a fruiting body at the end of development ($N_{ch}(t_f)$, t_f = post-development time-point) was calculated as the product of 3 variables: 1) initial total group size ($N(t_0)$), which includes both cooperator and cheater subpopulations and was varied from 10^2 to 10^6 , 2) the initial frequency of cheaters ($F_{ch}(t_0)$; 0.001, 0.01, 0.1, and 0.5), and 3) the frequency of cheater cells that convert to spores (D_{ch} ; Fiegna and Velicer 2005) which was varied from 0.001 to 1.

$$N_{ch}(t_f) = N(t_0) * F_{ch}(t_0) * D_{ch}$$

Semantics

Here, we use the term “cheating” to refer to a specific fitness relationship, namely exploitation of a strain with high performance at a focal social trait in pure culture (such as spore production during fruiting body formation) by a strain which performs poorly at the focal trait in pure culture when such exploitation confers a relative within-group fitness advantage to the low-performance strain (Kraemer and Velicer 2014). In this context, “exploitation” refers to a gain in the absolute performance of the cheater strain caused by interaction with the exploited strain. These definitions are not contingent on the molecular, behavioral or evolutionary causes of the respective phenotypes, which may not be known.

Statistics

All experiments were performed in at least 3 temporally independent replicate blocks and at least 12 fruiting bodies from each experimental population were examined. Statistical analysis of spores per fruiting body was performed with nonparametric tests using log₁₀-transformed data. Statistical tests were done with Prism v5.0 (GraphPad Software) and R v 3.1.2. Density distributions were based on 1d kernel estimates of the relative probabilities of fruiting bodies to contain particular numbers of spores as extrapolated from our observed data for the different strains tested here. Levene’s test for homogeneity of variance was performed using the “car” package for R. When relevant, *P* values were adjusted for multiple comparisons using the R function “p.adjust” and Bonferroni corrections.

RESULTS

Numbers of stress-resistant spores vary greatly across fruiting bodies

We first quantified the degree of variation in the number of heat- and sonication-resistant spores within individual fruiting bodies in a clonal population of laboratory strain GJVI. To do so, we estimated the number of spores within at least 12 individual mature fruiting bodies from 8 independent assays. Fruiting-body spore counts varied from roughly 30 individuals to $\sim 5 \times 10^5$ (Figure 1A; Supplementary Figure S1) across a largely continuous distribution (coefficient of variation = 19.2 after log₁₀-transformation). The large majority of fruiting bodies in most replicates contained between 10^3 and 10^4 spores, but one replicate each was strongly skewed toward the high and low ends of the distribution, indicating that minor uncontrolled variation in experimental conditions can strongly affect fruiting-body distributions in some cases. Across all replicates, the median ratio of largest/smallest spore counts per fruiting body was ~ 16 (Supplementary Table S2, Supplementary Figure S1). Nongenetic variation in group size was found to occur throughout the entire field of fruiting bodies, irrespective of distance from the field center, indicating that it is not merely a function of spatial position (Supplementary Figure S2).

Although after 5 days of development on starvation media all fruiting bodies are expected to be mature, we tested whether the observed nongenetic variation in spore numbers per fruiting body might be primarily due to differences in the developmental age of individual fruiting bodies. Sixteen hours after inoculation onto TPM plates, we visually selected fifteen of the earliest-forming nascent fruiting bodies from 4 independent populations of GJVI and analyzed them after 5 days of starvation. We then compared the range of spore estimates for these early-formed fruiting bodies and for 15 randomly chosen fruiting bodies within each replicate. The variance in spores per fruiting body did not consistently differ between early-forming and random fruiting bodies (Levene’s test, average *P* (across replicates) = 0.267), indicating that nongenetic variation in group size is prevalent even among the oldest class of fruiting bodies.

Modeling the formation of cheater-free fruiting bodies

Fruiting body development requires individually costly behaviors that can readily be exploited by socially defective cheaters (Velicer et al. 2000). Given that fruiting-body spore groups vary dramatically in size and some are very small, genetic variants (including cheater

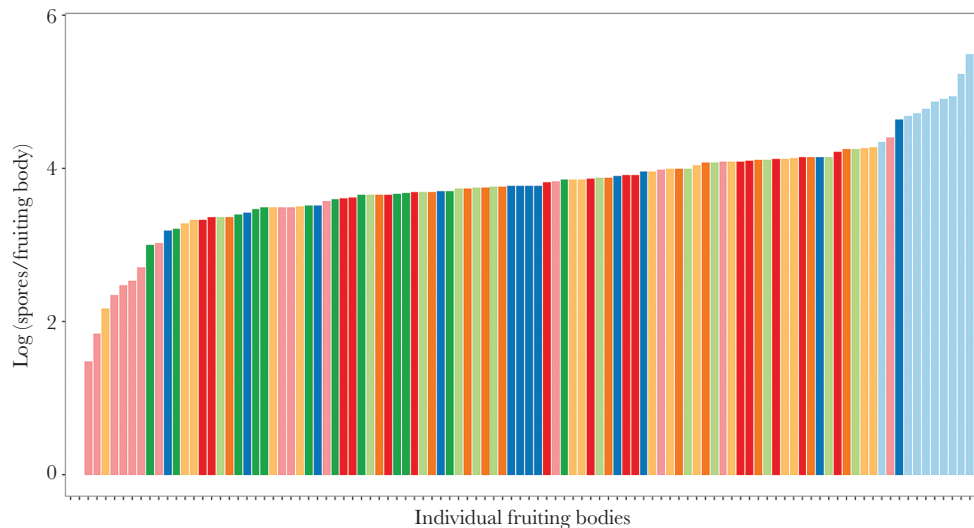


Figure 1

Nongenetic variation in spore number across discrete fruiting bodies in clonal populations. Numbers of spores (\log_{10}) per individual fruiting bodies across 8 independent replicates are shown. Color shading distinguishes replicate blocks.

genotypes) present as a minority of a local population at the initiation of starvation-induced development may not be represented among surviving spores in every fruiting body. To illustrate this, we modeled the absolute number of cheater-genotype spores expected to be found within individual fruiting bodies composed of only 2 genotypes, a developmentally proficient cooperator and a developmentally defective cheater. Cheater-spore numbers were calculated as a function of 3 variables: 1) initial group size, $N(t_0)$, (i.e. the total number of cells that initially aggregate into a fruiting body, irrespective of genotype), 2) the frequency of cheaters at the onset of development, $F_{ch}(t_0)$, and 3) the sporulation frequency of cheater cells (i.e. the frequency of cheater cells that survive starvation as spores), D_{ch} .

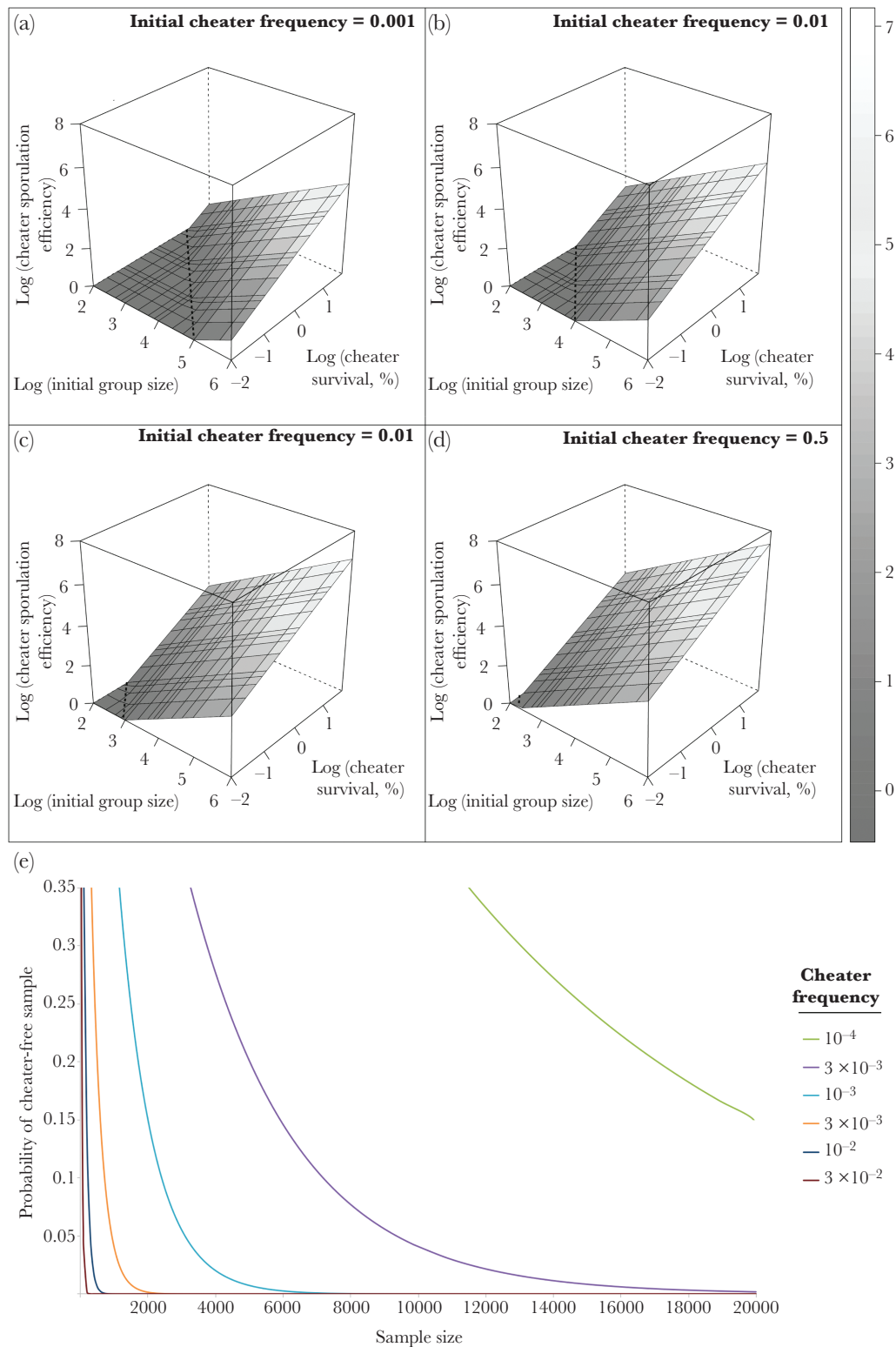
Across an entire population composed of many fruiting bodies, the proportion of developmentally proficient *M. xanthus* cells that survive development as stress-resistant spores in laboratory experiments often falls in the range of 1–10% (Licking et al. 2000) but can vary greatly across natural isolates and population densities (Kadam and Velicer 2006). The sporulation efficiency of cheater cells is often greater than that of cooperators and can reach 100% (Velicer et al. 2000). Given that most fruiting bodies in our initial assay contained from 10^3 – 10^4 spores whereas others had more or fewer, we varied initial group size, $N(t_0)$, (prior to sporulation) from 10^2 to 10^6 . We also varied cheater frequency from 0.001 to 0.5 and cheater survival rate from 0.001 to 1 (Figure 2A–D). Because sporulation efficiency is highly dependent on total population density (Kadam and Velicer 2006), it is plausible that at the fruiting-body level, sporulation efficiency varies with fruiting-body group size and may be lower within small fruiting bodies than in large ones.

At high initial cheater frequencies (e.g. 0.5, Figure 2D), only very small fruiting bodies combined with very low survival rates (e.g. an initial total group size 2×10^3 and a cheater survival frequency less than 0.001) would result in cheater-free fruiting bodies. However, cheater-free fruiting bodies are predicted for a range of smaller group sizes and lower initial cheater frequencies that might plausibly occur in actual fruiting bodies. For example, a group of 10 000 aggregating individuals in which cheaters initially compose 0.1% of the population (Figure 2A) and sporulate at a frequency of 0.05 has only a 50% chance of containing even a single cheater spore.

As an alternative approach, we also modeled random sampling from theoretical pools of already differentiated spores that include defined frequencies of cheater spores. Specifically, using a binomial distribution model in which sampling a cheater spore is a “success” and sampling a cooperator spore is a “miss,” we calculated the probability of sampling cheater-free spore sets as a function of sample size (analogous to fruiting body group size, based on our previous observations, Figure 1) and different cheater frequencies (Figure 2E). Although this model is not highly realistic biologically, it does illustrate the principle that random sampling readily generates cheater-free sample sets across a range of cheater-spore frequencies (Figure 2E). For example, for a cheater-spore frequency of ~ 0.001 , random samples of $N < \sim 5000$ total spores will generate cheater-free sample sets with a nonzero probability that increases as N decreases.

Small fruiting bodies are often cheater-free

To test whether any fruiting bodies in a cheater-infected population emerge from development cheater-free, we thoroughly mixed a developmentally proficient cooperator (GJV1) with a developmentally defective obligate cheater (OC; Velicer et al. 2000) at a 1:99 cheater:cooperator ratio and tested for the presence of OC in 15 individual fruiting bodies from 3 independent replicates. As was the case for pure cultures of GJV1, fruiting bodies in the chimeric population varied substantially in their total spore production (Figure 3A). On average across the whole population, strain OC cheated effectively by increasing its frequency 5-fold, despite its pure-culture defect. However, the number of OC spores within individual fruiting bodies varied more than the number of GJV1 spores, with OC variance consistently being greater than GJV1 variance across replicates (Figure 3B, Levene’s test, average P (across replicates) = 0.001). Importantly, no cheater spores were detected in approximately 10% of all fruiting bodies in this assay (5/45) at our limit of detection ($N = 5$; Figure 3A). These cheater-free fruiting bodies corresponded to those carrying lower numbers of total spores. Further, we observe a significant correlation between the number of total spores within a fruiting body

**Figure 2**

Models of cheater survival and random group sampling. The number of total surviving cheaters ($N_{ch}(t_f)$) per fruiting body is shown as a function of initial cheater-genotype frequency ($F_{ch}(t_0)$; (A), 0.001; (B), 0.01; (C), 0.1; (D), 0.5), initial total group size ($N(t_0)$) prior to spore differentiation and cheater sporulation efficiency (D_{ch}). All parameters except initial cheater frequency are \log_{10} -transformed. For plotting purposes, a default value of one cheater spore was used for parameter space in which the final number of cheaters per fruiting body is zero. (E) Binomial distribution of the probability of randomly sampling a cheater-free set of spores from a large population of cheater and cooperator spores as a function of total sample size and cheater frequency. This model is not biologically realistic, as it assumes random group formation after spore differentiation (and thus does not incorporate differences in sporulation efficiency, etc.), but it illustrates that even purely random sampling generates cheater-free groups. Values are plotted only within parameter space in which the product of total spore number (x-axis) and cheater-spore frequency is greater than 1.

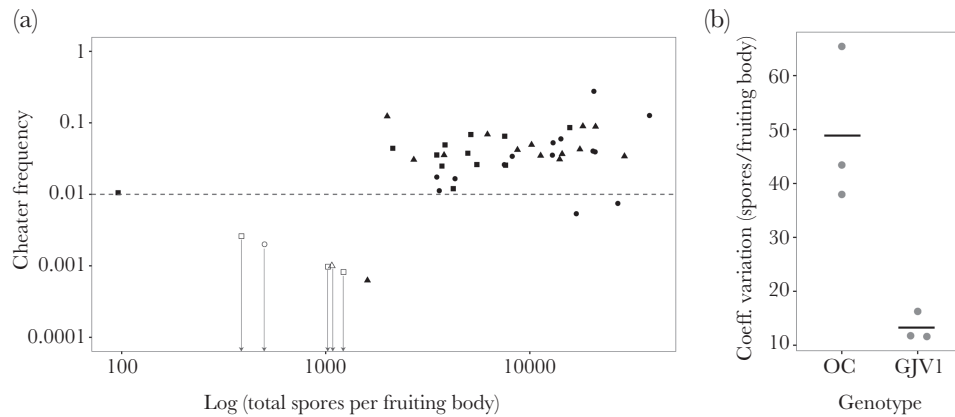


Figure 3

Nongenetic variation in group size results in cheater-free fruiting bodies. (A) Frequency of cheater spores within individual fruiting bodies (log scale). Each data point represents an individual fruiting body, the dashed line indicates the initial cheater frequency (0.01) prior to plating and starvation and the 3 symbols (dot, square, and circle) are used to distinguish 3 independent replicates. Open symbols with the downward pointing arrows indicate fruiting bodies in which no cheater spores were found at our limit of detection. For these cases, a cheater default value of 4 (just below the minimum detection level of the assay) was assigned for statistical purposes. (B) Coefficient of variation in the number of viable spores per fruiting body of each genotype. Dots represent independent replicates and horizontal bars represent means.

and the cheater frequency (Spearman's $\rho = 0.51$, $P = 0.00014$). To confirm that some fruiting bodies in fact do not carry any cheater spores at all, we directly plated 48 fruiting bodies from new co-developmental plates in selective media that allows only OC to grow. In this assay, 4 out of the 48 fruiting bodies did not grow into colonies, indicating a complete absence of OC spores.

Cooperator and cheater spore production decreases at high cheater frequency

It has been shown previously that when cheaters are present at high frequency they often impose cheating load, i.e. they reduce total group productivity (Velicer et al. 2000; Fiegna and Velicer 2003; Velicer and Vos 2009; Figure 3). Correspondingly, the individual sporulation efficiency estimates of both GJV1 and OC in 1:1 mixes are lower than at lower OC frequencies (Figure 4). Thus, although cheaters can increase within groups when they are at low frequency, they can decrease across groups when some groups have high frequencies of cheaters and others are cheater free.

Nonheritable group variation is widespread among natural isolates and across environments

We further tested whether the large degree of nongenetic variation in group size exhibited by the domesticated lab strain GJV1 is idiosyncratic to that strain or rather is a feature shared by most natural isolates of *M. xanthus* and other *Myxococcus* species. We, thus, estimated the numbers of spores within many fruiting bodies from each of 5 *M. xanthus* natural isolates (A47, A75, DK816, Mxx104, and Mxx144; Vos and Velicer 2009), one strain each of *Myxococcus flavescens* (Mxf1) and *Myxococcus virescens* (Mxv2) and a laboratory-evolved derivative of GJV1 (here referred to as GJV1*) which retained the ability to form viable spores and fruiting bodies (Supplementary Table S1). Individual fruiting body spores counts within each strain were found to also vary greatly for all additional strains (Figure 5A). Overall, ~60% of the variance observed was explained by effects of genotype, whereas 30% was due to within-genotype variation (linear mixed effects model). Finally, close visual inspection of the fruiting bodies formed by a genetically identical population also revealed marked morphological differences (Figure 5B-D). For instance, strain

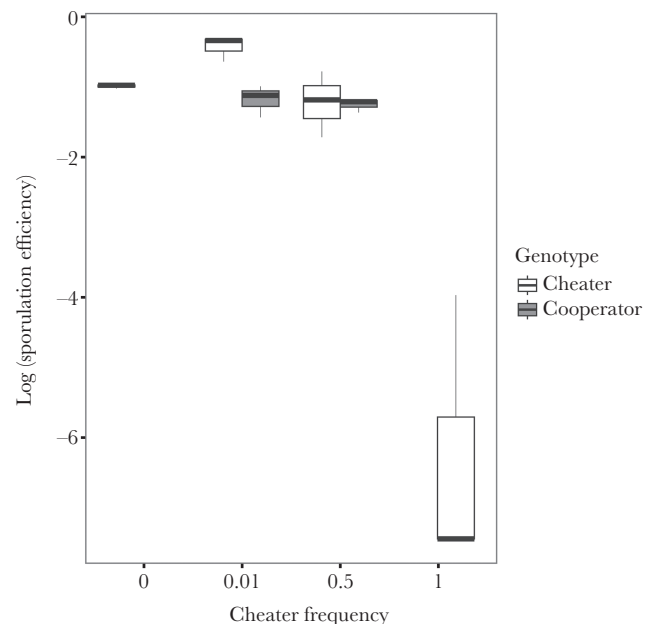


Figure 4

High frequencies of cheaters in mixed groups decrease the fitness of both cooperators and cheaters in mixed groups below the fitness of cooperators in pure groups. Sporulation efficiencies (frequencies of pre-development cells that become spores) in pure groups of cooperators (cheater frequency 0), mixed groups with cheaters initially mixed at frequencies of 0.01 and 0.5, and pure groups of cheaters (cheater frequency 1.0). At low frequency (0.01), cheaters have higher fitness than cooperators in mixed groups and cooperators in pure groups (cheater frequency 0), whereas at high frequency in mixed groups cheater fitness is approximately equal to cooperators in the same mixed groups but is lower than the fitness of cooperators in pure groups. Box plots represent the median and the lower and upper quartiles. Whiskers span 1.5 of the interquartile range from the lower and upper quartiles.

Mxx144 makes both heavily pigmented and lightly pigmented fruiting bodies and DK816 can form both small, round fruiting bodies or larger and elongated fruiting bodies (Figure 5B-D).

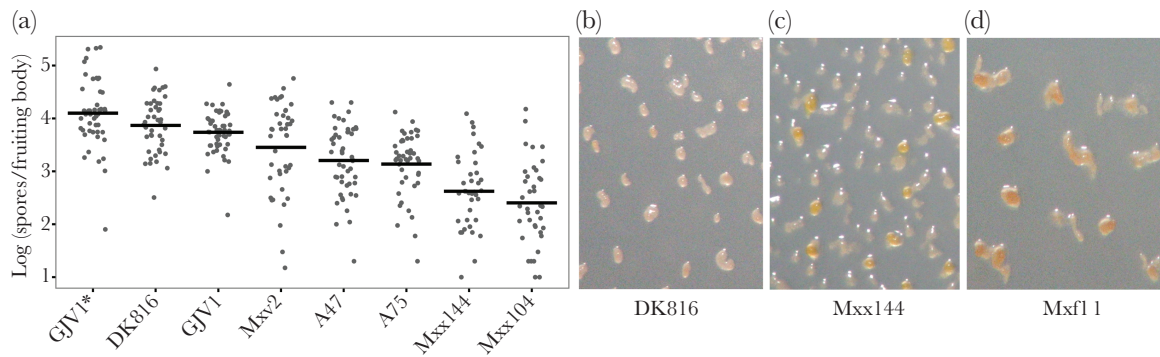


Figure 5

Variation in spore number per fruiting body occurs in multiple *Myxococcus* strains and species. (A) Log₁₀-transformed spores per fruiting body of different *Myxococcus* spp isolates. Each dot represents an individual fruiting body. Data from at least 3 independent experiments is presented. Horizontal bars represent means. Experiments were performed on TPM. (B–D) Representative pictures of developing populations. Strains DK816 and Mxx144 developed on TPM whereas *M. flavescens* strain Mxf11 developed on CF. All images were taken at the same scale.

The soil environments in which *Myxococcus* species live are extremely heterogeneous and fluctuating. We thus tested whether the nongenetic variation in fruiting-body spore numbers observed on complete starvation medium (TPM) is also observed in a different environment. To better mimic the presumably natural norm of groups growing and then starving in the same location, we repeated our experiments on CF medium, which contains a low concentration of nutrients that allows single cells to multiply and form colonies that deplete local nutrients and then form fruiting bodies (Rosenberg 1984). Nongenetic variation in individual fruiting-body spore counts was also found to occur on CF (Supplementary Figure S3A) to a similar or greater degree than that on TPM for all 3 strains tested (GJV1, GJV1*, and A47). Additionally, the probability distributions of fruiting-body spore counts differed significantly between CF and TPM (Kolmogorov–Smirnov test for difference in distributions: $P = 5.5 \times 10^{-7}$ for GJV1, $P = 0.004$ for GJV1*, and $P = 3.9 \times 10^{-5}$ for A47, Supplementary Figure S3B), indicating that environmental conditions can influence patterns of nongenetic variation in group size. Taken together, our results indicate that nongenetic variation in group size across fruiting bodies (as reflected by viable spore counts) is a general feature of *Myxococcus* development that is qualitatively robust across diverse genotypes and environments.

Patterns of group-level phenotypic variation vary heritably across *Myxococcus* isolates

A previous study documented extensive heritable variation in developmental life-history traits across *M. xanthus* isolates at the population level, but did not investigate traits at the fruiting body level (Kraemer et al. 2010). We tested whether particular patterns of nonheritable fruiting-body variation within genetically homogeneous populations might themselves vary heritably across *Myxococcus* genotypes. First, fruiting body morphology varied greatly across strains (Figure 5B–D). Second, the number of individual fruiting bodies formed by the different strains from the same initial number and density of cells also varied significantly (e.g. 476 on average for DK816 vs. 3300 for Mxx104, Kruskal–Wallis, $\chi^2 = 18.67$, $df = 7$, $P = 0.002$, Supplementary Figure S4, post hoc Supplementary Table S3). Finally, the average number of spores per fruiting body (Figure 5A, Kruskal–Wallis test, $\chi^2 = 24.16$, $P < 0.0001$, post hoc Supplementary Table S4) also differed across strains. Taken together, some group-level traits such as fruiting body morphology, number of fruiting bodies formed, and the average number of

spores within fruiting bodies vary heritably across *Myxococcus* strains and species.

DISCUSSION

Fruiting body development is presumably a major component of overall fitness in natural populations of myxobacteria. In *M. xanthus*, development has been extensively studied from a variety of perspectives, including its underlying molecular mechanisms (Kroos 2007), morphogenesis dynamics (Kaiser and Welch 2004; Holmes et al. 2010), the evolution of developmental genes (Chen et al. 2014), and heritable natural variation across genotypes isolated at a wide range of spatial scales (Kraemer et al. 2010; Kraemer and Velicer 2011), but variation across distinct fruiting bodies composed of the same genotype in the same population has received little attention. It is commonly quoted that $\sim 10^5$ cells aggregate into *M. xanthus* fruiting bodies (Shimkets 1990; Zusman et al. 2007). However, we have shown that fruiting bodies in a clonal population vary greatly in spore number, by up to a factor of $\sim 10^4$, which suggests that they also vary greatly in the initial number of aggregating cells. Ultimately, our results show that organismal behavior naturally generates variation in group size, which in turn generates variation in the bottleneck size of local groups, including the formation of small groups that are cheater-free, even when cheaters are present at readily detectable and increasing frequencies in a broader population of larger fruiting bodies.

Theoretical and experimental studies have shown that population bottlenecks reduce within-group competition and help maintain microbial social traits (Brockhurst 2007). Bottlenecks, alongside other mechanisms such as social barriers to migration between groups (Vos and Velicer 2009; Rendueles et al. 2015a; Rendueles et al. 2015b), selective sweeps, and founder effects, can increase relatedness within groups and limit the spread of noncooperative genotypes that have the potential to drive a population to extinction when the cooperative trait is important for survival (Fiegna and Velicer 2003). In the case of multicellular fruiting body development, recent work with the social amoeba *Dictyostellium discoideum* has shown that repeated experimental imposition of the most extreme bottleneck possible (i.e. a single cell) allows proficiency at cooperative development to persist over many generations due to a low rate of mutation to developmentally defective cheating phenotypes (Kuzdzal-Fick et al. 2011). This experiment is analogous to

the production of single-cell gametes by multicellular eukaryotes, which eliminates within-soma genetic variation and thereby eliminates intercellular evolutionary conflicts (Grosberg and Strathmann 1998; Ostrowski and Shaulsky 2009). Such extreme bottlenecks are important drivers of evolution, natural genetic diversity and speciation events (Travisano and Velicer 2004; Brockhurst 2007; Abel et al. 2015) and promote the stabilization of multicellular cooperation (Foster et al. 2004; Griffin et al. 2004; Travisano and Velicer 2004).

Our results show that nongenetic variation in the natural process of group formation in an aggregatively multicellular microbe has the potential to similarly purge genetic conflict by reducing the bottleneck size of some groups (Figure 6). This may have implications for cooperator-cheater dynamics in the wild (Figure 6). Cheater-free fruiting bodies generated by this process can then germinate to establish new cheater-free groups of vegetative cells, either from the location at which the fruiting body initially formed (if new growth substrates arrive there) or in a new location if the fruiting body is

dispersed by wind, water, or animals (Figure 6). In contrast, large fruiting bodies are more likely to retain cheaters that have invaded a local population, which can then continue to increase in frequency in some group lineages until they cause local decreases of cooperation due to cheating load, and possibly even local extinction events (Fiegna and Velicer 2003; Fiegna et al. 2006; Figure 6).

Thus, our data suggest that nongenetic variation in group size may be one mechanism, amongst others (Velicer et al. 2000; Velicer and Vos 2009; Manhes and Velicer 2011; Rendueles et al. 2015b), that stabilizes cooperation in natural *M. xanthus* populations by promoting statistically preferential interactions among cooperators. The formation of cheater-free groups would increase the potential for selection at the level of whole fruiting-body performance to limit long-term equilibrium frequencies of cheaters, because cooperator fitness in pure groups is higher than either cooperator or cheater fitness in groups that contain high frequencies of cheaters and thus suffer cheating load (Figures 4 and 6; Velicer and Vos 2009). The principle that such group-level selection can prevent cheaters from

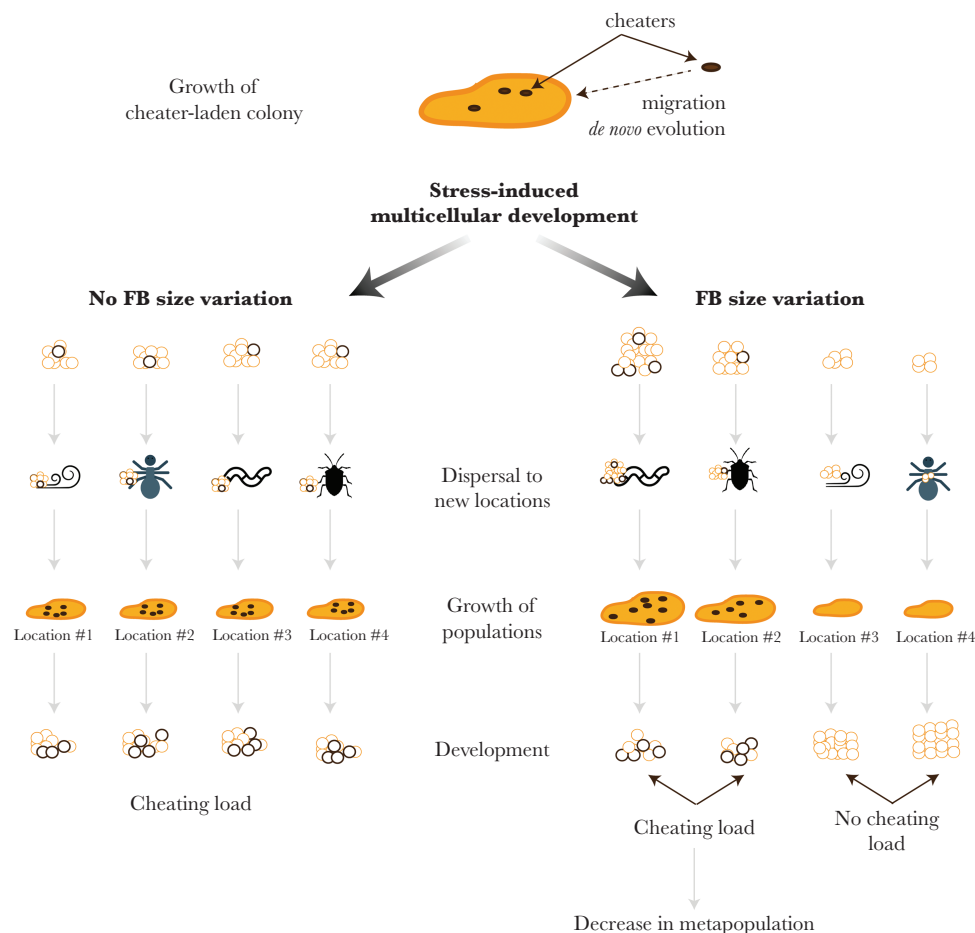


Figure 6

Cheater-free group formation and the *Myxococcus* life cycle. *M. xanthus* is a soil-dwelling motile microbe that upon starvation or other stressful conditions aggregates to form mounds that will in turn develop into fruiting bodies. Inside the latter, a minority of cells differentiate into metabolically quiescent spores that are resistant to heat and desiccation, whereas the majority remain as undifferentiated rods or lyse. Because myxobacterial development is a cooperative trait, it is subject to cheating. Developmental cheaters (depicted in brown) in *M. xanthus* are often unable to produce fruiting bodies when in clonal groups but outperform cooperators (depicted in orange) at sporulation within mixed groups. Such cheaters can emerge de novo within a population or migrate from other neighboring social groups. After maturation, fruiting bodies may be passively disseminated to new locations by various vectors, and once conditions become favorable for growth, spores subsequently germinate and establish new vegetative colonies. In cheater-laden populations, cheaters could increase in number in subsequent cycles of development, reduce the overall productivity of the fruiting bodies in which they are common (relative to fruiting bodies formed from cheater-free colonies) and thus fail to fix in the larger metapopulation.

driving cooperators to extinction in a metapopulation (Simpson's paradox) has been illustrated with a synthetic microbial system (Chuang et al. 2009). However, an assessment of the degree to which nongenetic variation in fruiting-body group size influences the levels of cooperation in natural populations of *M. xanthus* will require greater knowledge of myxobacterial behavior in the wild.

An expected consequence of generating small groups is an increase of the local role of genetic drift relative to larger groups (Futuyama 2009). Our results show that cheaters may fail to be represented in all small fruiting bodies even when the overall social environment should, in theory, select for their increase. Analogously, small group-size bottlenecks increase the role of chance in determining the local fates of genetic variation more broadly, potentially leading to local increases of non-advantageous alleles or decreases of adaptive alleles.

Several considerations suggest that the nongenetic variation in group size documented here might underestimate the frequency of very small fruiting bodies in wild populations. First, our fruiting-body harvesting procedure was biased toward picking spatially isolated fruiting bodies to ensure that only one fruiting body was picked at a time. Second, most of the experiments were performed on TPM agar, an environment lacking growth substrates, whereas more complex and heterogeneous environments might more accurately mimic the situation for natural populations. Third, we excluded from analysis all sonicated fruiting bodies from which no colonies germinated after dilution, which would have also excluded any fruiting bodies that contained fewer spores than our statistical limit of detection (4 for pure culture populations).

There are several caveats to the interpretation of our results. First, we know little about fruiting body formation in natural populations. For example, we have no data on the frequency at which *M. xanthus* engages in development and/or the reversibility of cell fates at different stages of development or relationships between different degrees and types of stress (e.g. starvation, exposure to antibiotics, etc.) and cell death across extremely complex natural habitats. Throughout this study (as in most studies of *M. xanthus* development), we assume for simplicity that the differentiation state of cells is bimodal, with each cell either transforming into a stress-resistant spore or not and correspondingly surviving or dying. However, it is unclear whether all vegetative cells would die under most natural conditions that induce fruiting body development. It is possible that physiological states among cells that are not fully resistant to the heat and sonication stress we imposed to select spores may represent a continuum of resistance to various degrees of stress (e.g. nutrient level, duration of starvation, etc.). To the degree that this is the case, the average size of viable cell groups may be somewhat larger than estimated here for natural conditions that are less stressful than standard experimental treatments for selecting spores. Nonetheless, our results show that under at least some conditions of stress, small cheater-free groups can emerge within a broader cheater-infected population. More broadly, given the lack of understanding of in situ natural populations, here we only suggest, based on laboratory experiments, that cheater-free fruiting-body formation may contribute to the stabilization of cooperation but make no claims regarding the importance of this mechanism relative to other processes that are likely to promote preferential interaction among cooperators.

As one final caveat, the methods employed here did not allow accurate estimates of how many cells initially aggregate into an

individual fruiting body prior to spore differentiation. We, therefore, assumed that sporulation efficiency across different fruiting bodies from the same population is constant. However, this might not be the case. Just as sporulation efficiency correlates with population density at the whole-population level across many fruiting bodies, within-fruiting body sporulation efficiency may correlate positively with group size. If this is the case, small fruiting bodies would make proportionally fewer total spores than large ones, thus decreasing the bottleneck size of viable spores further than might be predicted from the aggregate size alone.

The causes of nongenetic variation of group size remain to be explored and the biophysical parameters determining variation in the spatio-temporal dynamics of developmental aggregation and fruiting body formation are still poorly understood (Xie et al. 2011). A plausible default null hypothesis is that fruiting-body group size is an intrinsic property of myxobacterial development resulting from the physical and spatial dynamics of cellular migration, interaction, and aggregation in structured environments and that selection does not act on the degree or distribution of variation in group size per se. The observation of nongenetic variation in group size in all examined natural isolates is consistent with this hypothesis, as is the retention of ancestral degrees of nongenetic variation in group size in a lab strain (GJV1*) that underwent extensive evolution in the absence of selection for developmental proficiency (Rendueles et al. 2015b). Under this hypothesis, an intrinsic characteristic of fruiting body development (as opposed to an adaptively evolved characteristic) occasionally generates small group sizes that result in group-level cheater loss.

We do not propose that the propensity of *M. xanthus* fruiting bodies to vary nongenetically in group size is favored by selection specifically for the evolutionary purpose of recurrently generating small cheater-free groups. However, we do draw a limited analogy to forms of nongenetic variation at the individual level (or phenotypic heterogeneity) that have often been interpreted as adaptive (Viney and Reece 2013). Nongenetic phenotypic variation among bacterial cells can generate phenotypes that are detrimental to the fitness of some individuals in some environments but which would be beneficial to those same individuals in other environments. Thus, among genetically identical individuals, the stochastic generation of multiple phenotypes has often been viewed as beneficial for fitness at the lineage level and has thus been termed bet hedging (Veening et al. 2008). For example, some bacteria and yeast generate genetically identical cells that vary greatly in growth rate (Levy et al. 2012). Although the generation of slow-metabolism cells may appear to be disadvantageous, in fact such persister cells can be more tolerant to antibiotics than fast-growing cells (Balaban et al. 2004) and thus have a fitness advantage in some environments.

Analogously, in microorganisms that form distinct social groups, group characteristics that are detrimental in some respects may have unexpected positive effects as well. For example, individuals in a small fruiting body may often be at a disadvantage to others in large fruiting bodies because upon spore germination, *M. xanthus* growth is thought to be density-dependent in many environments (Rosenberg et al. 1977). However, in the event of a local cheater invasion (whether caused by a local mutation event or migration), individuals entering smaller fruiting bodies would be less likely to co-aggregate with cheater cells into a common fruiting body and thus they (or their descendants) might avoid cheating load (Velicer and Yu 2003).

Finally, if the recurrent regeneration of small, cheater-free groups occurs in wild populations, it would shift the relative degree

of selection that occurs across versus within fruiting body groups and thus promote the maintenance of aggregative multicellularity. Taken together, our results highlight the value of examining the evolutionary significance of all forms of biological variation, both heritable and nonheritable, at all levels of biological organization.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at <https://academic.oup.com/beheco/>

AUTHOR CONTRIBUTIONS

M.A. performed experiments. G.J.V. designed experiments and composed the manuscript. O.R. designed and performed experiments, analyzed data and composed the manuscript.

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by (Amherd et al. 2017) in the Dryad Digital Repository.

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