

Research



Cite this article: Larsen TJ, Jahan I, Brock DA, Strassmann JE, Queller DC. 2023 Reduced social function in experimentally evolved *Dictyostelium discoideum* implies selection for social conflict in nature. *Proc. R. Soc. B* **290**: 20231722.
<https://doi.org/10.1098/rspb.2023.1722>

Received: 31 July 2023

Accepted: 21 November 2023

Subject Category:

Evolution

Subject Areas:

evolution, microbiology

Keywords:

Dictyostelium discoideum, experimental evolution, cheating, relaxed selection, sociobiology, social amoebae

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Electronic supplementary material is available online at <https://doi.org/10.6084/m9.figshare.c.6961023>.

Reduced social function in experimentally evolved *Dictyostelium discoideum* implies selection for social conflict in nature

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Many microbes interact with one another, but the difficulty of directly observing these interactions in nature makes interpreting their adaptive value complicated. The social amoeba *Dictyostelium discoideum* forms aggregates wherein some cells are sacrificed for the benefit of others. Within chimaeric aggregates containing multiple unrelated lineages, cheaters can gain an advantage by undercontributing, but the extent to which wild *D. discoideum* has adapted to cheat is not fully clear. In this study, we experimentally evolved *D. discoideum* in an environment where there were no selective pressures to cheat or resist cheating in chimaeras. *Dictyostelium discoideum* lines grown in this environment evolved reduced competitiveness within chimaeric aggregates and reduced ability to migrate during the slug stage. By contrast, we did not observe a reduction in cell number, a trait for which selection was not relaxed. The observed loss of traits that our laboratory conditions had made irrelevant suggests that these traits were adaptations driven and maintained by selective pressures *D. discoideum* faces in its natural environment. Our results suggest that *D. discoideum* faces social conflict in nature, and illustrate a general approach that could be applied to searching for social or non-social adaptations in other microbes.

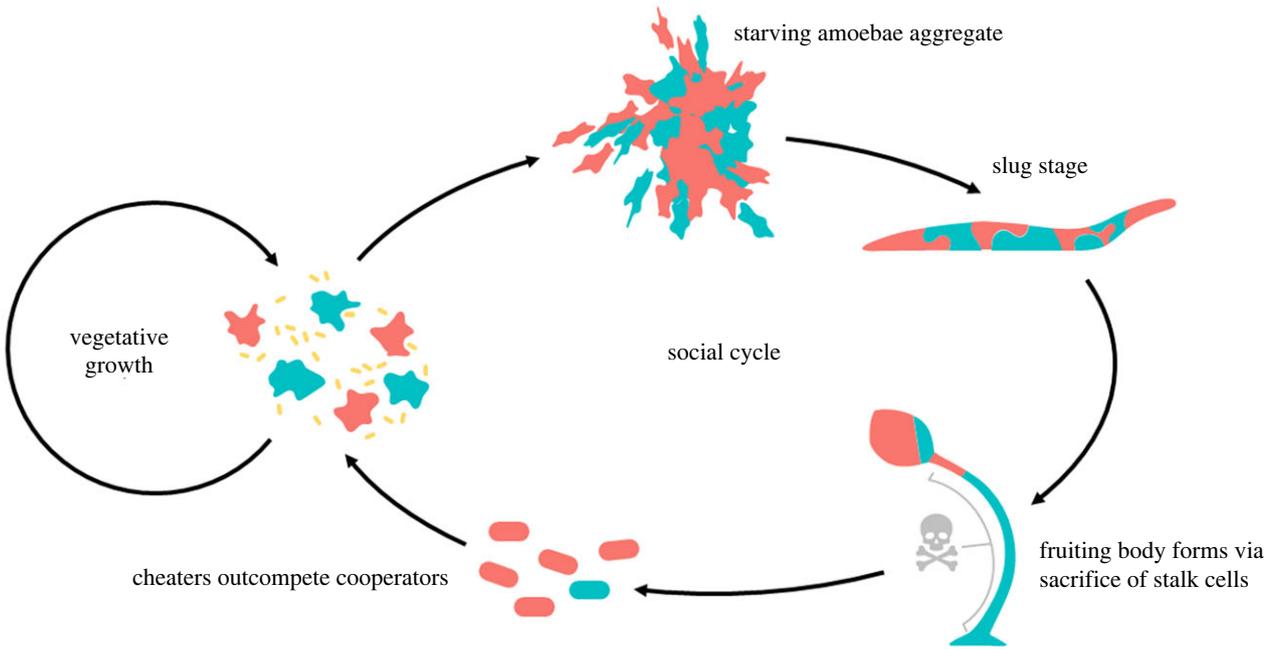
1. Introduction

Microbes are capable of social behaviour that once would have seemed beyond the abilities of such tiny organisms. Despite their small size and simplicity, microbes can cooperate to sense their environment [1–3], hunt prey [4], kill enemies [5], protect friends [6], move over difficult ground [7], collect nutrients [8] and more. Microbial cooperation is worthy of study in its own right for its significant consequences for human health and on the ecological services microbes provide, but also because microbes have proven to be uniquely valuable model organisms for studying major questions about evolution [9–11].

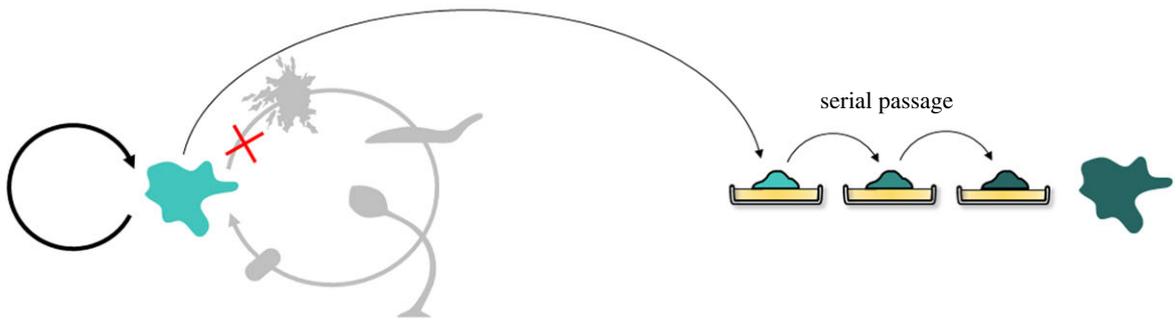
One long-standing question of interest is how cooperation evolves and is maintained despite the threat of exploitation by non-cooperating cheaters [12–16]. Many functions that larger organisms perform privately microbes must perform publicly through the production and secretion of molecules into their environment, which would seem to render them especially vulnerable to the risk of exploitation. This, combined with their short generations and high population sizes, makes social microbes especially well-suited for studying the evolution of cooperation and conflict.

Dictyostelium discoideum is an interesting eukaryotic microbe with utility as a model organism for scientists studying development, multicellularity, immunology, cooperation and conflict [17–19]. While it spends most of its life as a solitary unicellular hunter of bacteria, when starving, *D. discoideum* enters a multicellular life stage (figure 1) [19–21]. Local amoebae aggregate together to form a slug-like multicellular body that can move towards light. Upon reaching a suitable spot, the slug develops into a fruiting body consisting

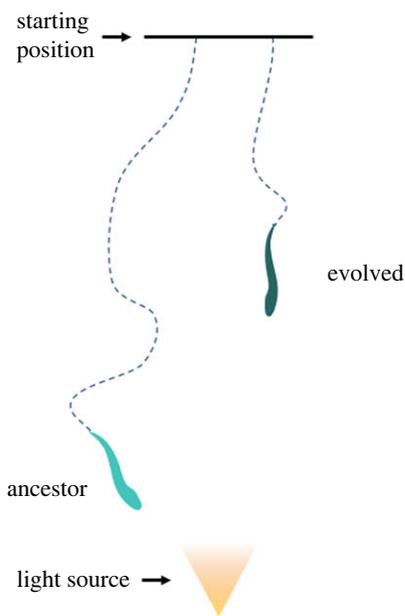
(a) *D. discoideum* social cycle allows apparent cheating



(b) experimental evolution



(c) assaying slug stage mobility



(d) assaying cheating

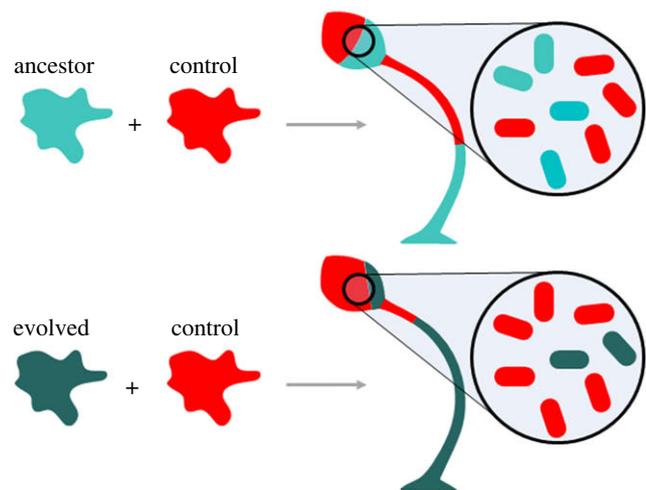


Figure 1. (Caption continued.)

Figure 1. (Continued.) Experiment overview. (a) Simplified schematic of *D. discoideum*'s social cycle, in which multiple genotypes (red and teal) can potentially aggregate into chimaeric multicellular bodies. Most of the time, amoebae are unicellular and grow vegetatively. Upon starvation, cells aggregate and develop into a multicellular slug stage, which moves towards light, and then a sessile fruiting body. The formation of the fruiting body requires the sacrifice of a minority of cells to produce a stalk, and so cheater genotypes (red) can gain an advantage by undercontributing to stalk formation. Cheating appears to provide clear benefits in artificial settings but its relevance to wild *D. discoideum* is debated. (b) Experimental evolution of *D. discoideum* under conditions which prevent it from entering the social cycle should drive the loss of traits previously maintained by selective pressures related to it. (c) Slug mobility assays—experimentally evolved *D. discoideum* (dark green) should travel less far during the slug stage than its ancestor (teal) due to relaxed selection. (d) Cheating assays—if cheating is adaptive in nature, experimentally evolved *D. discoideum*, when mixed equally into a chimaera with an RFP-labelled control strain (red), should be less well represented among the spores of the resulting fruiting body than its ancestor due to relaxed selection.

of a ball-shaped sorus of durable spores held aloft by a stalk of dead somatic cells. The spores wait dormant in the sorus to be dispersed—possibly by a passing invertebrate—to a new location with sufficient prey [22,23].

Dictyostelium discoideum aggregates form from local amoebae, which do not need to be closely related [18,24,25]. When cells aggregate into chimaeras containing multiple unrelated cell lineages, they have the opportunity for conflict that cells within more conventional multicellular organisms—made up of the clonal descendants of a single cell—mostly avoid. In *D. discoideum*, this conflict centres on the production of its fruiting body's characteristic stalk, which requires about 20% of the cells within an aggregate to die to help disperse the remainder. Such a sacrifice would not be remarkable within a clone (picture the staggering majority of human cells that toil and die just to pass on a few gametes), but in a chimaeric aggregate, it creates an incentive for competing cell lineages to under-contribute to stalk production to minimize their own losses and exploit more cooperative lineages [18,26,27].

Multiple studies have observed cheating in *D. discoideum* [18,28–31]. *Dictyostelium discoideum* chimaeras form readily in the laboratory, and careful observation of the fates of cells within them reveals that not every cell lineage contributes equally to stalk production. Some *D. discoideum* strains appear to be consistently prone to exploiting or prone to being exploited by others [28,32] such that a simple hierarchy of cheaters and cooperators can be determined. Studies have identified mutations that cause strains to cheat [29–31,33], and in experimental evolution experiments where *D. discoideum* is allowed to mutate, mix and fruit, non-fruiting obligate cheaters readily evolve and outperform cooperative strains, even to the point of causing population crashes [34]. Some evidence suggests that *D. discoideum* has mechanisms for maintaining high relatedness within fruiting bodies, which may imply the existence of adaptations to reduce the risk of exploitation by maximizing the chance that cells aggregate with cooperative kin. There is evidence that *D. discoideum* actively segregates between kin and non-kin at least temporarily during aggregation [35,36], though to what extent kin discrimination may protect against cheaters is unclear [37–39]. Nonetheless relatedness within fruiting bodies in nature is very high [24], probably owing to population structure imposed by the way *D. discoideum* grows and disperses [40,41]. Signatures of the frequency-dependent selection that often accompanies evolutionary conflict have been detected in genes known to affect cheating [42]. *D. discoideum* was also found to recognize and respond to the presence of non-kin within a chimaeric aggregate with changes in gene expression, development and dispersal behaviour relative to clonal aggregates [43].

Despite multiple lines of evidence, however, some researchers have questioned the relevance of cheating to wild *D. discoideum*. As with any trait, directly proving that

cheating is an adaptation is difficult, particularly when most studies of *D. discoideum* cheating involve laboratory-made chimaeras. Non-fruiting obligate cheaters readily arise and prosper (at least over the short term) in experimental evolution experiments using *D. discoideum* under conditions imposing low relatedness [34], but none were observed in a screen of 1039 spores isolated from 75 wild-collected fruiting bodies [24]. This may suggest that in nature, the benefits obligate cheaters reap by reducing stalk production in chimaeras are too small or too infrequently realized to compensate for the disadvantages of being unable to produce a functioning stalk when there are no cooperative lineages to exploit—*D. discoideum* cheaters in nature are likely to be facultative cheaters. Results of genomic studies of *D. discoideum* genes differentially expressed in chimaeras have been mixed—one study observed signatures of increased polymorphism and rapid evolution consistent with evolutionary conflict [44], while another did not [45]. In the absence of direct observation of cheating in nature, some researchers have proposed that apparent cheating is a laboratory artefact of little relevance to wild *D. discoideum*, and better explained by variation in non-social life-history traits [46–48]. In this, cheating in *D. discoideum* is in good company with other well-supported social traits in microbes that have faced similar skepticism [49,50].

In this study, we sought to infer whether or not *D. discoideum*'s apparent cheating was the result of adaptations to selective pressure to cheat (or resist cheating) in nature. We experimentally evolved multiple wild *D. discoideum* strains under conditions in which they never entered the social stage of their lifecycle and thus never had the opportunity to cheat. Without the opportunity to aggregate, selection on social traits like cheating should be relaxed, generally leading to losses in social function normally maintained by natural selection.

Key to this approach is the assumption that when a long-standing selective pressure is removed, past adaptations driven by that pressure are likely to be lost due to drift or, more likely, pleiotropic trade-offs with other traits. Selection upon one trait will often indirectly impact other traits, and an organism's traits often represent a compromise between mutually incompatible adaptations to different selective pressures. These compromises should tend to constrain the evolution of adaptations to overcome any particular selective pressure. An organism cannot, for example, evolve to be smaller to save energy and larger to avoid predation at the same time. If one selection pressure is relaxed, however, compromises are no longer necessary and constraints are lifted. For this reason, we should expect that relaxing a selective pressure should free an organism to lose traits that were adaptations to that pressure [51–54].

We can look for these sorts of losses and use them to infer selective pressures (and resultant adaptations) that we

hypothesize are important but that are difficult to directly observe, such as the pressure that wild *D. discoideum* would experience if they were regularly exploited by cheaters. When *D. discoideum* is evolved in an environment where we know cheating is not relevant, it should lose adaptations related to cheating, but it cannot lose adaptations that it does not have. Therefore, loss of function when we make social conflict irrelevant in the laboratory is evidence that we have successfully relaxed a selective pressure that was relevant in nature. By contrast, if cheating in *D. discoideum* is the result of selective pressures on other, non-social traits and only appears to be cheating in an artificial laboratory environment, relaxation of selective pressures on the social stage should not affect it.

In this study, we experimentally evolve *D. discoideum* under conditions where cheating cannot occur, then measure the effects of experimental evolution on *D. discoideum*'s ability to cheat during the formation of chimaeric fruiting bodies. In addition, we assayed the effects of experimental evolution on two other phenotypes to act as controls with which we could validate our logic. First, as an example of a trait that we were very confident experienced relaxed selection in our experiment, we assayed the distance travelled by *D. discoideum* slugs after aggregation but prior to fruiting body formation. The complexity of slug formation and migration, and its ability to enable aggregates to move towards the soil surface and form fruiting bodies where they are in the best position to be dispersed, is likely to be adaptive for *D. discoideum* in its natural environment [55]. In laboratory conditions where no slugs are formed, however, slug migration is irrelevant or even potentially maladaptive if there are trade-offs between it and more useful traits. We expect slug migration distance to be uncorrelated, or negatively correlated with, fitness in the laboratory and so we expect to see our evolved *D. discoideum* lines evolve reduced migration distance relative to their ancestors.

As an example of a trait that we are confident would not experience relaxed selection in our experiment, we assayed the number of cells *D. discoideum* produced within 48 h. Cell population is a product of traits—like growth rate and efficient use of resources—that we expect to be under strong selection both in nature and in the laboratory. In fact, the simplicity, mildness and abundance of the laboratory environment is likely to result in increased selection to produce more cells more quickly relative to a natural environment full of abiotic and biotic threats and scarcity. We thus expect total cell production to be positively correlated with fitness in the laboratory, and do not expect to see loss of function in these traits—in the laboratory, our *D. discoideum* still use it, so they will not lose it. Together slug migration and cell production traits act as controls because they reflect a selective pressure that we experimentally relaxed and one that we did not relax, respectively. By comparing the effects of experimental evolution on cheating with its effects on these traits, we should be able to infer whether *D. discoideum* experiences selection pressure to cheat in nature.

2. Methods

(a) Culture conditions

We performed experimental evolution using SM/5 media [56] (2 g glucose (Fisher Scientific), 2 g BactoPeptone (Oxoid), 2 g

yeast extract (Oxoid), 0.2 g MgCl₂ (Fisher Scientific), 1.9 g KH₂PO₄ (Sigma-Aldrich), 1 g K₂HPO₅ (Fisher Scientific) and for solid media 15 g agar (Fisher Scientific) per litre deionized water). To start a fresh culture of *D. discoideum*, we diluted spores from –80°C glycerol frozen stocks in KK2 buffer (2.25 g KH₂PO₄ (Sigma-Aldrich) and 0.67 g K₂HPO₄ (Fisher Scientific) per litre deionized water). We plated 1.0×10^5 total spores onto an SM/5 plate along with 200 µl *K. pneumoniae* food bacteria resuspended in KK2 to an OD₆₀₀ of 1.5. To start a fresh culture of any bacterial strain, we streaked stocks from the minus 80°C freezer for isolation on SM/5 plates. We performed slug migration and spore production assays on nutrient-free starving agar plates.

(b) Antibiotic curing of *D. discoideum*

Many wild *D. discoideum* isolates are infected by *Paraburkholderia* symbionts which affect their fitness and behaviours [57]. To remove symbionts, 1.0×10^5 *D. discoideum* spores of each clone were plated on SM/5 agar medium containing 30 µg ml⁻¹ tetracycline and 10 µg ml⁻¹ ciprofloxacin with 200 µl of *K. pneumoniae* resuspended in KK2 to an OD₆₀₀ of 1.5. We allowed plates to grow at room temperature under ambient light until formation of fruiting bodies (3–5 days). We collected spores as above, then diluted and plated again as above. We then collected spores and performed spot test assays (described in [58]) and PCR using *Paraburkholderia*-specific primers [59] to verify successful curing.

(c) Experimental evolution

Three replicate lines each of ten strains (electronic supplementary material, table S1) were plated on SM/5 plates. We incubated all lines at room temperature under ambient light and transferred 0.5% of the population to fresh plates every 48 h. We performed transfers by first harvesting all cells into 10 ml KK2 buffer using gentle pipetting and scraping of the agar surface. We then thoroughly vortexed the resulting suspensions, diluted them 200-fold and plated 100 µl onto fresh plates with 200 µl of an OD₆₀₀ = 1.5 *K. pneumoniae* suspension to serve as food. The 48 h transfer interval was selected to preempt *D. discoideum*'s fruiting stage and prevent direct selection on social traits. Every fifth transfer, we additionally froze 1 ml of the undiluted suspension of harvested cells at –80°C with 60% glycerol.

Following experimental evolution over 30 transfers, *D. discoideum* lines were checked for cross-contamination using fragment analysis. We extracted DNA from 100 µl of the undiluted suspension of harvested cells using CHELEX resin beads and amplified using fluorescently tagged PCR primers specific to highly variable microsatellite loci known to differ in length between *D. discoideum* strains [60]. Fragment analysis of resulting amplicons was performed by Genewiz and evolved strains were compared to ancestors. No cross-contamination was detected.

(d) Cheating assays

To assay cheating, we determined the proportion of fluorescent spores in fruiting bodies developing from an initial 50:50 mix of a strain of interest and the RFP-labelled control strain RFP-NC28.1. We assayed cheating in chimaeras comprising lines of interest and a labelled control strain to reflect the natural context where a cheater must compete against an unrelated strain. For each ancestor and evolved line, we plated 2×10^5 frozen spores onto an SM/5 agar plate. Inocula for evolved lines represented a genetically diverse population. During mid-log stage (approx. 34–36 h after plating), we collected vegetative cells and washed three times with cold KK2 buffer, counted using a hemacytometer and diluted each suspension to 10⁸ cells ml⁻¹. We combined equal

volumes of the focal strain and the labelled control strain and gently mixed to get 50:50 mix suspensions. We prepared UV-sterilized 13mm² AABP 04700 (Millipore) filter squares. We pipetted 15 µl of the 50:50 mix suspension into the centre of each of three filter squares pre-dampened with KK2. We transferred filters onto KK2 (non-nutrient) agar plates to initiate immediate aggregation and development and incubated plates for 5 days at room temperature.

After incubation, we examined and selected two filters from each plate to assay. We prioritized filters where all fruiting bodies formed on the filter (that is, filters where no slugs had escaped onto the surrounding agar, leading to artificially lower total spores present)—otherwise selection was random. We collected each filter with the fruiting bodies that had grown atop them with sterile forceps into 500 µl KK2 buffer, vortexed thoroughly and took photographs with brightfield and fluorescence microscopy. We captured at least five fields for each sample (representing around 1000–2000 spores). Each assay was performed three times to make three technical replicates (independent plates/assays of cells from the same frozen stock) for each of the ten ancestors and thirty experimentally evolved lines.

We counted total spores and fluorescent spores using Fiji [61]. We first manually converted micrographs into binary images using a brightness threshold set individually for each image to account for minor differences in contrast and brightness between samples. We then used Fiji's Count Particles function, filtering for particles between 15 and 200 µm² and between 0.5 and 1.0 circularity (settings which consistently resulted in very similar results to manual counting).

We determined proportion of fluorescent spores by dividing the number of fluorescent spores (count from fluorescent image) by the total spores (count from brightfield image). We also determined a percentage fluorescence for the RFP-NC28.1 control for each assay and divided this proportion out of the results for the 50:50 mix samples in order to compensate for incomplete labelling.

(e) Slug migration assays

In order to compare how experimental evolution affected slug migration distance, we performed assays on ancestral and evolved lines. Each assay was performed three times to make three technical replicates (separate plates/assays of cells from the same frozen stock) for each of the 10 ancestors and thirty experimentally evolved lines. For each assay, we marked a 10 cm secant line on a 13 cm non-nutrient agar plate. We plated 50 µl of an OD₆₀₀ = 50.0 suspension of *K. pneumoniae* in KK2 buffer containing 10⁷ *D. discoideum* spores along the secant line. Inocula for evolved lines represented a genetically diverse population. We allowed the loaded sample to dry and wrapped the plates individually in aluminium foil. On each wrapped plate, we made a small pin-hole opposite the starting line through which light could enter. We placed the wrapped plates on the laboratory bench under a light source and left them undisturbed for 8 days. At the end of the 8 days, we unwrapped the plates and photographed using a Canon EOS 5D Mark III camera.

We used Fiji [61] to perform image processing and obtain slug migration distances. First, we scaled images and overlaid a 1 cm × 1 cm grid. We marked fruiting bodies on each image and measured their distance from the starting line.

(f) Cell count assays

To assay cell populations, we performed assays on ancestral and evolved lines. For each ancestral and evolved line, we plated 1 × 10⁵ frozen spores on a fresh SM/5 nutrient agar plate, along with 200 µl of an OD₆₀₀ = 1.5 suspension of *K. pneumoniae* food bacteria. Inocula for evolved lines represented a genetically diverse population. Plates were incubated at room temperature. After 48 h, all cells were collected into a suspension of KK2 buffer by

pipetting buffer onto each plate's surface and gently agitating with a pipette tip. The resulting suspensions were then vortexed and counted using a hemocytometer to determine total cell populations on each plate. Conditions, timing and process of cell count assays were identical to those used during the experimental evolution process. Each assay was performed three times to make three technical replicates (separate plates/assays of cells from the same frozen stock) for each of the 10 ancestors and 30 experimentally evolved lines.

(g) Spore count and sporulation efficiency assays

We assayed spore production using the plates prepared for the slug migration assays. One day after plates from the slug migration assays were imaged, we collected the fruiting bodies from each plate into KK2 buffer. We counted the spores within the resulting suspensions under a light microscope using a hemocytometer.

To test the possibility that apparent reductions in cheating were the result of differences in the efficiency of sporulation of evolved *D. discoideum* lines, we also performed spore production assays on ancestral and evolved lines on plates without food bacteria. For each ancestral and evolved line, we plated 1.5 × 10⁶ amoebae onto UV-sterilized 13 mm² AABP 04700 (Millipore) filter squares on a fresh non-nutrient KK2 plate without exogenous food. Inocula for evolved lines represented a genetically diverse population. Under these conditions, amoebae immediately starve and enter the fruiting stage. After 96 h, we collected each filter into a tube with 500 µl KK2 buffer, vortexed and counted using a hemocytometer to determine the total spore production of each plate. By immediately inducing the fruiting stage and leaving no opportunity for growth, this assay effectively isolates differences in the efficiency with which *D. discoideum* lines could convert amoebae into spores. Each assay was performed three times to make three technical replicates (separate plates/assays of cells from the same frozen stock) for each of the 10 ancestors and 30 experimentally evolved lines.

(h) Statistical analysis

We performed analyses using R version 4.0.4 (R Core Team) [62] with the *lme4* package [63] and the *emmeans* package [64].

All statistical analyses were performed using the mean of three technical replicates. To analyse changes in spore production and slug migration distance, we used linear mixed effects models with treatment (ancestor versus evolved) as a fixed effect and strain as a random effect. The model used to analyse slug migration was Average Migration Distance ~ Treatment + (1 | Strain/Line), where Treatment was a 2-level factor (Ancestral or Evolved), Strain represented which of the 10 ancestor lineages were being tested (QS6, QS9, QS18, QS70, QS159, QS161, QS11, QS69, QS395, QS859), and Line represented which replicate evolved line was being tested (E1, E2 or E3). In the slug migration assays, in order to account for fruiting bodies that developed at the starting line (migration distance = 0), we weighted each plate's contribution to the model by the fraction of spores from fruiting bodies with non-zero migration distances. This had the effect of reducing the average migration distance on each plate by the fraction of spores whose slugs did not migrate beyond the starting zone. The model used to analyse cell count and spore count data were Total Counted ~ Treatment + (1 | Strain/Line).

To analyse changes in representation among spores in chimaeric fruiting bodies, we used a two-tailed, two-proportion Z-test to maximize our statistical power. We also analysed these data using a linear mixed effect model (Proportion of spores ~ Treatment + (1 | Strain/Line)) and observed qualitatively similar statistically significant results (not reported).

In order to convey the most biologically meaningful insight, we have reported our results in terms of 95% confidence intervals in addition to *p*-values [65].

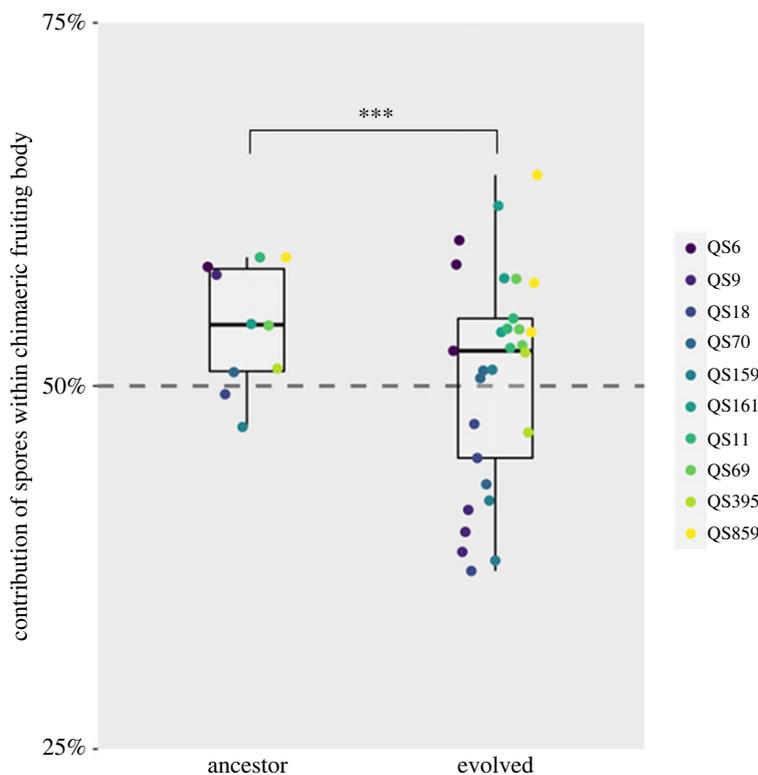


Figure 2. Experimentally evolved *D. discoideum* evolve reduced ability to cheat (or resist being cheated upon). Spores of ancestral *D. discoideum* strains are overrepresented within chimaeric fruiting bodies made by combining equal numbers of the focal strain and a labelled control strain. When competed against the same control strain, evolved *D. discoideum* strains contribute a lower percentage of spores within fruiting bodies compared to their ancestors, suggesting that experimental evolution resulted in reduced cheating ability (or reduced ability to resist being cheated upon). Each point represents the average of three technical replicates.

3. Results

(a) Cheating assays

For each *D. discoideum* line, we combined amoebae of the line of interest with amoebae of the control strain RFP-NC28.1 at a 50:50 ratio, then measured the proportion of spores within the resulting chimaeric fruiting bodies belonging to the control strain. Ancestral *D. discoideum* strains made up an average of 54.27% of spores in chimaeric fruiting bodies, suggesting that overall the wild strains could initially socially outcompete RFP-NC28.1. In chimaeras made from evolved *D. discoideum* strains and RFP-NC28.1, evolved *D. discoideum* strains were significantly less well represented than their ancestors had been ($\chi^2_1 = 175.34$, $p < 0.001$), contributing an average of 51.07% of spores. Possible values for the true decrease most compatible with our data ranged from -2.65% to -3.56% (95% CI) (figure 2).

The reduced representation of evolved lines among spores within chimaeras was not the result of evolved lines being less capable of producing spores than their ancestors. We did not detect a significant difference in sporulation efficiency between amoebae from ancestral and evolved *D. discoideum* lines inoculated clonally on SM/5 plates without exogenous food ($t_{28.09} = 0.421$, $p = 0.677$). Under these conditions, which should eliminate any effect of differences in vegetative cell fitness between lines, ancestral *D. discoideum* produced an average of 9.31×10^5 spores per plate after 4 days. Experimentally evolved *D. discoideum* produced an average of 9.06×10^5 (-2.7%) spores per plate over the same timeframe. Possible values for the true change most compatible with our data ranged from 8.38×10^5 to 1.08×10^6 (-10.1% to $+15.9\%$) (95% CI) (electronic supplementary material, figure S1).

(b) Slug mobility assays

We measured the effects of experimental evolution on slug migration by assaying the average distance travelled by slugs produced by ancestral and evolved lines from a starting position. Ancestral *D. discoideum* slugs migrated an average of 4.01 cm from the starting line before fruiting. Experimentally evolved *D. discoideum* slugs migrated less far, moving an average of 3.52 cm (-12.2%) less far than their ancestors ($t_{27.49} = -2.969$, $p = 0.00367$). Possible values for the true reduction in migration distance most compatible with our data ranged from 0.82 cm to 0.17 cm (-20.4% to -4.2%) (95% CI) (figure 3b).

(c) Cell count assays

We measured the effects of experimental evolution on *D. discoideum*'s ability to reproduce in the laboratory by comparing the total cell populations reached by ancestral and evolved lines in 48 h. Ancestral *D. discoideum* reached an average population of 1.9×10^8 cells per plate after 48 h. Experimentally evolved *D. discoideum* produced an average of 5.0×10^7 ($+26.3\%$) more cells than their ancestors within the same timeframe ($t_{29} = 2.366$, $p = 0.0249$). Possible values for the true increase most compatible with our data ranged from 8.5×10^6 to 9.1×10^7 ($+4.5\%$ to $+47.9\%$) (95% CI) (figure 4).

4. Discussion

Dictyostelium discoideum is a useful model organism for studying cooperation and conflict. Its social cycle requires potentially unrelated cells to cooperate, but also creates incentive for cells

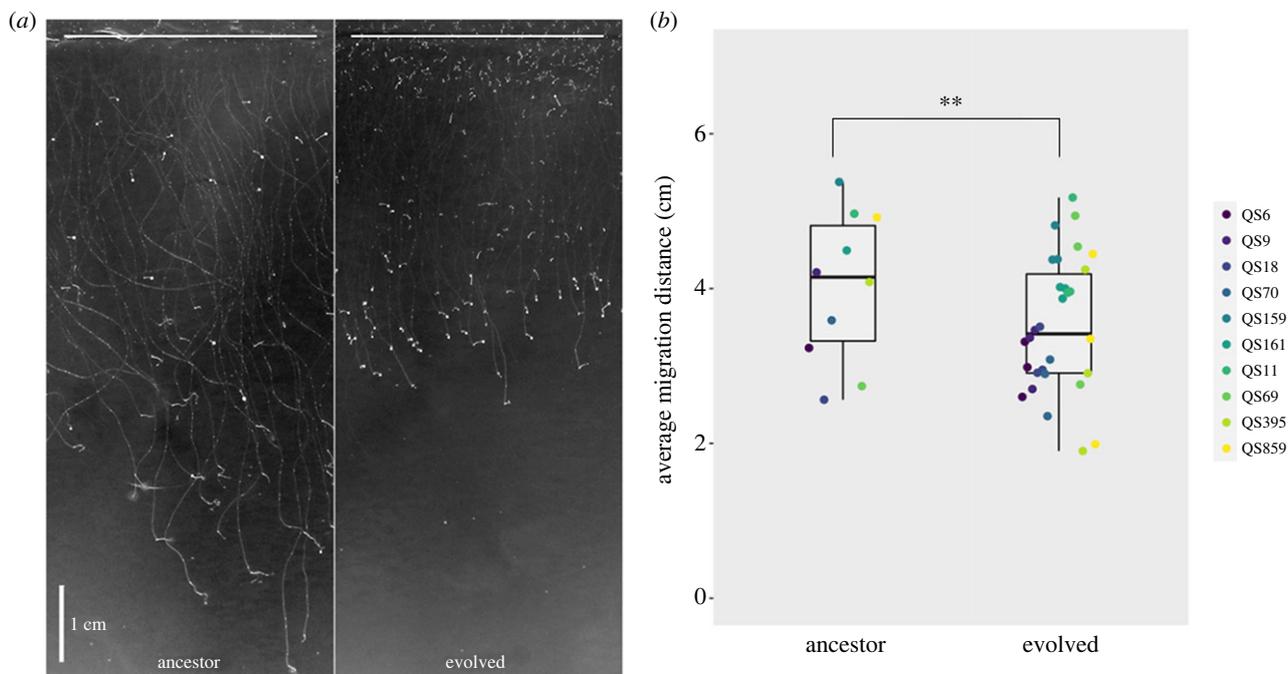


Figure 3. Experimentally evolved *D. discoideum* evolve reduced slug mobility. (a) Representative image of slug mobility assays. (b) *D. discoideum* strains evolved without the opportunity to aggregate migrate shorter distances during the slug stage than their ancestors. Each point represents the average of three technical replicates.

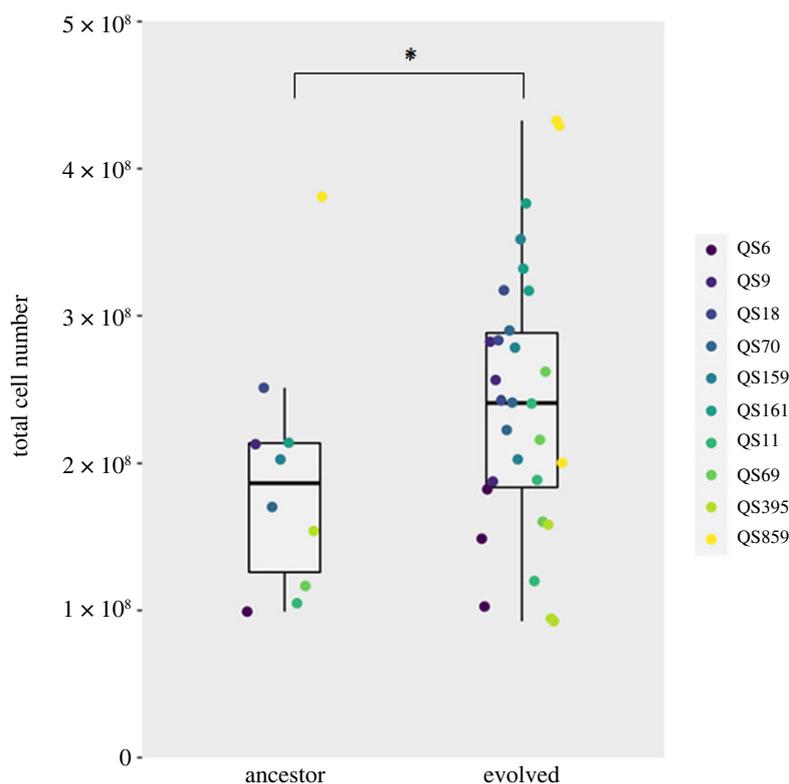


Figure 4. Experimentally evolved *D. discoideum* strains produce more cells—*D. discoideum* strains evolved without the opportunity to aggregate produce significantly more cells than their ancestors within a 48 h period. Each point represents the average of three technical replicates.

to exploit one another. While multiple lines of evidence attest to some *D. discoideum* strains' ability to exploit one another in the laboratory, interpreting the relevance of cheating to *D. discoideum* in nature is not trivial. In this respect, cheating in *D. discoideum* echoes other prominent examples of microbial sociality [49,50] and illustrates a general challenge in studying adaptations in any organism. It is easier to prove that an

organism has a particular trait than it is to be certain what selective pressures (if any) drove the trait's evolution, and doubly so if the organism is too small to directly observe in its natural habitat.

This study attempts to shed light on the adaptive value of cheating in *D. discoideum* in nature by experimentally evolving wild strains of *D. discoideum* under laboratory conditions in

which the social cycle—and thus cheating—is prevented. This experiment hinges on the idea that relaxing selective pressures an organism faces in nature will tend to result in loss of adaptations maintained by those pressures. Organisms seem to ‘use it or lose it’ and become less well adapted to environments in which they do not live [51,52]. For example, vestigial or transient traits like the hindlimbs of cetaceans, reduced digits in birds and ungulate mammals, and the eyes of cave fish appear to serve little function but may be the remnants of adaptations to past environments where selective pressures were different [53,66,67]. Bacteria that live inside larger organisms, and in particular intracellular endosymbionts, experience high rates of decay and loss of genes that are essential for free-living bacteria but not necessary inside of a host [68,69]. Similarly, organisms adapt to selective pressures imposed by other organisms with which they interact and lose these adaptations when their biotic context changes [70]. Viruses that infect an unfamiliar host tend to lose their ability to infect their original host, allowing for the creation of attenuated vaccines [71]. Island animals freed from the threat of predation lose anti-predator behaviours maintained by those on the mainland [72–74]. Further, trait losses have frequently been observed in experimental evolution experiments, wherein animal or microbial populations are often passaged in much simpler environments than those in which they evolved [75–82].

We looked for changes in experimentally evolved *D. discoideum*'s ability to cheat that would suggest it had experienced selective pressure to cheat (or resist cheating) in its natural environment. In addition, we assayed slug migration distance and cell number, two phenotypes for which we had opposite *a priori* predictions.

We found that compared to their ancestors, experimentally evolved *D. discoideum* were less capable cheaters (or, alternatively, less capable of resisting being cheated upon) within chimaeric fruiting bodies (figure 2). This result is what we would expect if our ancestral *D. discoideum* strains had adaptations to make them better cheaters (or more resistant to being cheated upon) that atrophied when we relaxed the selective pressures that were maintaining them.

This interpretation is strengthened by our results for slug migration and cell number, which act positive and negative controls, respectively, for our method. Slugs produced by experimentally evolved *D. discoideum* lines migrated less far than those produced by their ancestors (figure 3). As slug migration is certainly an adaptive trait in nature, we expected our ancestral lines to have adaptations that enhanced it. Accordingly, when we relaxed selective pressure on these adaptations by preventing slug formation entirely, we expected to see evolved lines form less functional slugs for the same reason we are suggesting that they became less functional cheaters. By contrast, we found that evolved *D. discoideum* produced more cells than their ancestors (figure 4), probably as the result of selection for higher growth rates and/or more efficient use of resources in the laboratory during the vegetative stage. This reflects that the laboratory environment—where food is plentiful and conditions are mild—obliges cells to invest in outcompeting neighbours rather than protect themselves from no-longer-relevant hazards that they would face in nature. Cell number remained at least as relevant during our experimental evolution experiment as it would have been in nature, and thus we did not expect the reduction in this trait that we observed in the cheating and slug migration distance traits.

One potential alternative explanation for the reduced representation of evolved lines within chimaeras in our cheating assays could be due to changes in sporulation efficiency. If evolved lines simply became worse at producing spores from starved cells, they would contribute fewer spores in chimaeric mixtures. This would appear like a loss of cheating (or cheating resistance) but for completely non-social reasons. To test this possibility, we assayed sporulation efficiency (the fraction of a population of starved amoebae that become spores) directly. We observed no difference between ancestral and evolved lines (electronic supplementary material, figure S1a). Ultimately, our evolved lines are at least as good at producing spores as their ancestors, and (due to higher cell numbers produced during the vegetative stage) often better (electronic supplementary material, figure S1b). Since this would tend to make them appear to be *better* cheaters in our cheating assays, we suspect that the reduction in cheating we observed is, if anything, an underestimate, and that the best explanation is that we were observing losses of adaptations no longer being maintained by selection.

A similar concern arises in interpreting the results of our slug migration assays. We observed higher cell numbers in our evolved lines, presumably due to selection on vegetative cell fitness. But does cell number impact slug migration directly? Past research has demonstrated that when plated at higher densities, *D. discoideum* aggregates produce larger slugs which travel longer distances [83]. We largely account for this problem by performing these assays using standardized inocula of starving amoebae on non-nutrient plates, thus standardizing the number of amoebae we start with and minimizing the opportunity for vegetative growth during the assay. Any remaining growth opportunities due to, for example, uneaten bacteria travelling with the inocula could still impact the slug size and migration distance of high-fitness evolved lines, but should lead to *increased* migration distance rather than the decrease we observed. Thus, again, we expect that the reduced slug migration distance of our evolved lines is if anything an underestimation of the true extent of loss of slug functionality experienced due to evolving under relaxed selection.

Adaptations are not free, and adapting to enhance one trait can come at the expense of unrelated traits that may be adaptive for other reasons [84–86]. While positive pleiotropic interactions exist between some traits, it is probably more common for unrelated functions to trade off negatively for the same reason that detrimental mutations are more common than beneficial mutations—there are more ways to break a system than there are to improve it [52,87]. Traits which serve different adaptive functions may trade off antagonistically—either because the functions themselves are less than fully compatible or simply because they represent competing uses of an organism's limited resources—and thus trait values may sometimes represent some balance between competing constraints. The need to compromise between traits can lead to apparent maladaptation and potentially constrain evolution [88,89]. While the difficulties of measuring pleiotropy make direct interrogation of this phenomenon complicated [89–91], there exists a growing body of evidence that antagonistic pleiotropy may be a key factor in the evolution of life-history traits like senescence [92–94] and in the maintenance of apparently maladaptive disease alleles [95,96].

If *D. discoideum* has adaptations that enhance its ability to cheat (or resist being cheated), they are likely to come at some

cost to adaptations to other selective pressures. When *D. discoideum* is experimentally evolved under conditions where it is no longer selected to cheat, these trade-offs should incentivize it to lose cheating adaptations. By contrast, if cheating is not selected for in nature, *D. discoideum* should not have adaptations supporting it, and moving cells to an environment where cheating continues not to be adaptive should not change anything.

The results of our cheating assays suggest that wild *D. discoideum* does have adaptations related to social conflict which they lost when evolved in an artificial environment in which cheating was necessarily irrelevant to their fitness. The removal of any selective pressure on the social stage freed experimentally evolved lines to evolve based upon non-social selective pressures alone. Our results are consistent with past molecular evolution studies implying *D. discoideum* genes affecting cheating have an evolutionary history driven by social conflict [42,44]. *Dictyostelium discoideum* probably does cheat in nature.

Microbes lead complicated lives obscured from us by their small size. Understanding even some apparently central aspects of their biology is a complex task demanding multiple approaches and careful interpretation. *Dictyostelium discoideum*'s social cycle has been the subject of interest for decades, and yet how exactly it fits into this microbe's life in nature continues to inspire debate. The results of this study support the idea that some wild *D. discoideum* strains experience enough social conflict to have evolved adaptations

to it. Further, the approach we have employed here—looking for otherwise inscrutable adaptations by evolving them away in laboratory—should be applicable to a wide variety of traits in a wide variety of organisms. This approach can usefully supplement other approaches in researchers' pursuit of a more complete understanding of adaptation.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Data available from the Dryad Digital Repository [97]. Supplementary material is available online [98].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. T.J.L.: conceptualization, formal analysis, investigation, project administration, writing—original draft, writing—review and editing; I.J.: formal analysis, investigation; D.A.B.: investigation, methodology, supervision; J.E.S.: conceptualization, funding acquisition, project administration, supervision, writing—review and editing; D.C.Q.: conceptualization, funding acquisition, project administration, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This study is supported by the National Science Foundation under grant nos. IOS 1656756 and DEB 1753743 and DEB 2237266.

Acknowledgements. We thank members of the Queller/Strassmann lab, Jonathan Losos, Carlos Botero and Fred Inglis for helpful discussion during the writing of this paper.

References

- Nealson KH, Hastings JW. 1979 Bacterial bioluminescence: its control and ecological significance. *Microbiol. Rev.* **43**, 496–518. (doi:10.1128/mr.43.4.496-518.1979)
- Fuqua WC, Winans SC, Greenberg EP. 1994 Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **176**, 269–275. (doi:10.1128/jb.176.2.269-275.1994)
- Abreu F, Martins JL, Silveira TS, Keim CN, De Barros HGPL, Filho FJG, Lins U. 2007 'Candidatus Magnetoglobus multicellularis', a multicellular, magnetotactic prokaryote from a hypersaline environment. *Int. J. Syst. Evol. Microbiol.* **57**, 1318–1322. (doi:10.1099/ijs.0.64857-0)
- Velicer GJ, Vos M. 2009 Sociobiology of the myxobacteria. *Annu. Rev. Microbiol.* **63**, 599–623. (doi:10.1146/annurev.micro.091208.073158)
- Riley MA, Wertz JE. 2002 Bacteriocins: evolution, ecology, and application. *Annu. Rev. Microbiol.* **56**, 117–137. (doi:10.1146/annurev.micro.56.012302.161024)
- Vidakovic L, Singh PK, Hartmann R, Nadell CD, Drescher K. 2018 Dynamic biofilm architecture confers individual and collective mechanisms of viral protection. *Nat. Microbiol.* **3**, 26–31. (doi:10.1038/s41564-017-0050-1)
- Kearns DB. 2010 A field guide to bacterial swarming motility. *Nat. Rev. Microbiol.* **8**, 634–644. (doi:10.1038/nrmicro2405)
- Saha R, Saha N, Donofrio RS, Bestervelt LL. 2013 Microbial siderophores: a mini review. *J. Basic Microbiol.* **53**, 303–317. (doi:10.1002/jobm.201100552)
- Ratcliff WC, Denison RF, Borrello M, Travisano M. 2012 Experimental evolution of multicellularity. *Proc. Natl Acad. Sci. USA* **109**, 1595–1600. (doi:10.1073/pnas.1115323109)
- Lenski RE, Rose MR, Simpson SC, Tadler SC. 1991 Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am. Nat.* **138**, 1315–1341. (doi:10.1086/285289)
- Lenski RE, Travisano M. 1994 Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc. Natl Acad. Sci. USA* **91**, 6808–6814. (doi:10.1073/pnas.91.15.6808)
- Hamilton WD. 1963 The evolution of altruistic behavior. *Am. Nat.* **97**, 354–356. (doi:10.1086/497114)
- Axelrod R, Hamilton WD. 1981 The evolution of cooperation. *Science* **211**, 1390–1396. (doi:10.1126/science.7466396)
- Maynard-Smith J. 1964 Group selection and kin selection. *Nature* **201**, 1145–1147. (doi:10.1038/2011145a0)
- Williams GC. 2018 *Adaptation and natural selection: A critique of some current evolutionary thought*, Vol. **75**. Princeton, NJ: Princeton University Press.
- Dawkins R. 2006 *The selfish gene*, 30th anniversary edition. Oxford, UK: Oxford University Press.
- Annesley SJ, Fisher PR. 2009 *Dictyostelium discoideum*—a model for many reasons. *Mol. Cell. Biochem.* **329**, 73–91. (doi:10.1007/s11010-009-0111-8)
- Strassmann JE, Zhu Y, Queller DC. 2000 Altruism and social cheating in the social amoeba *Dictyostelium discoideum*. *Nature* **408**, 965–967. (doi:10.1038/35050087)
- Kessin RH. 2001 *Dictyostelium: evolution, cell biology, and the development of multicellularity*, vol. **38**. Cambridge, UK: Cambridge University Press.
- Bonner JT. 1944 A descriptive study of the development of the slime mold *Dictyostelium discoideum*. *Amer. J. Bot.* **31**, 175–182. (doi:10.1002/j.1537-2197.1944.tb08016.x)
- Strassmann JE, Queller DC. 2011 Evolution of cooperation and control of cheating in a social microbe. *Proc. Natl Acad. Sci. USA* **108**, 10 855–10 862. (doi:10.1073/pnas.1102451108)
- Smith J, Queller DC, Strassmann JE. 2014 Fruiting bodies of the social amoeba *Dictyostelium discoideum* increase spore transport by *Drosophila*. *BMC Evol. Biol.* **14**, 105. (doi:10.1186/1471-2148-14-105)
- Huss MJ. 1989 Dispersal of cellular slime molds by two soil invertebrates. *Mycologia* **81**, 677–682. (doi:10.1080/00275514.1989.12025808)
- Gilbert OM, Foster KR, Mehdiabadi NJ, Strassmann JE, Queller DC. 2007 High relatedness maintains

- multicellular cooperation in a social amoeba by controlling cheater mutants. *Proc. Natl Acad. Sci. USA* **104**, 8913–8917. (doi:10.1073/pnas.0702723104)
25. Ostrowski EA. 2019 Enforcing cooperation in the social amoebae. *Curr. Biol.* **29**, R474–R484. (doi:10.1016/j.cub.2019.04.022)
26. Armstrong DP. 1984 Why don't cellular slime molds cheat? *J. Theor. Biol.* **109**, 271–283. (doi:10.1016/S0022-5193(84)80006-5)
27. Buss LW. 1982 Somatic cell parasitism and the evolution of somatic tissue compatibility. *Proc. Natl Acad. Sci. USA* **79**, 5337–5341. (doi:10.1073/pnas.79.17.5337)
28. Fortunato A, Queller DC, Strassmann JE. 2003 A linear dominance hierarchy among clones in chimeras of the social amoeba *Dictyostelium discoideum*. *J. Evol. Biol.* **16**, 438–445. (doi:10.1046/j.1420-9101.2003.00545.x)
29. Khare A, Shaulsky G. 2010 Cheating by exploitation of developmental prestalk patterning in *Dictyostelium discoideum*. *PLoS Genet.* **6**, e1000854. (doi:10.1371/journal.pgen.1000854)
30. Santorelli LA, Kuspa A, Shaulsky G, Queller DC, Strassmann JE. 2013 A new social gene in *Dictyostelium discoideum*, chtB. *BMC Evol. Biol.* **13**, 1–10. (doi:10.1186/1471-2148-13-4)
31. Ennis HL, Dao DN, Pukatzki SU, Kessin RH. 2000 *Dictyostelium* amoebae lacking an F-box protein form spores rather than stalk in chimeras with wild type. *Proc. Natl Acad. Sci. USA* **97**, 3292–3297. (doi:10.1073/pnas.97.7.3292)
32. Buttery NJ, Rozen DE, Wolf JB, Thompson CRL. 2009 Quantification of social behavior in *D. discoideum* reveals complex fixed and facultative strategies. *Curr. Biol.* **19**, 1373–1377. (doi:10.1016/j.cub.2009.06.058)
33. Santorelli LA *et al.* 2008 Facultative cheater mutants reveal the genetic complexity of cooperation in social amoebae. *Nature* **451**, 1107–1110. (doi:10.1038/nature06558)
34. Kuzdzal-Fick JJ, Fox SA, Strassmann JE, Queller DC. 2011 High relatedness is necessary and sufficient to maintain multicellularity in *Dictyostelium*. *Science* **334**, 1548–1551. (doi:10.1126/science.1213272)
35. Ostrowski EA, Katoh M, Shaulsky G, Queller DC, Strassmann JE. 2008 Kin discrimination increases with genetic distance in a social amoeba. *PLoS Biol.* **6**, e287. (doi:10.1371/journal.pbio.0060287)
36. Benabentos R *et al.* 2009 Polymorphic members of the lag gene family mediate kin discrimination in *Dictyostelium*. *Curr. Biol.* **19**, 567–572. (doi:10.1016/j.cub.2009.02.037)
37. Strassmann JE. 2016 Kin discrimination in *Dictyostelium* social amoebae. *J. Eukaryot. Microbiol.* **63**, 378–383. (doi:10.1111/jeu.12307)
38. Ho H-I, Hirose S, Kuspa A, Shaulsky G. 2013 Kin recognition protects cooperators against cheaters. *Curr. Biol.* **23**, 1590–1595. (doi:10.1016/j.cub.2013.06.049)
39. Kundert P, Shaulsky G. 2019 Cellular allorecognition and its roles in *Dictyostelium* development and social evolution. *Int. J. Dev. Biol.* **63**, 383. (doi:10.1387/ijdb.190239gs)
40. Buttery NJ, Jack CN, Adu-Oppong B, Snyder KT, Thompson CRL, Queller DC, Strassmann JE. 2012 Structured growth and genetic drift raise relatedness in the social amoeba *Dictyostelium discoideum*. *Biol. Lett.* **8**, 794–797. (doi:10.1098/rsbl.2012.0421)
41. Smith J, Strassmann JE, Queller DC. 2016 Fine-scale spatial ecology drives kin selection relatedness among cooperating amoebae. *Evolution* **70**, 848–859. (doi:10.1111/evo.12895)
42. Ostrowski EA *et al.* 2015 Genomic signatures of cooperation and conflict in the social amoeba. *Curr. Biol.* **25**, 1661–1665. (doi:10.1016/j.cub.2015.04.059)
43. Noh S, Christopher L, Strassmann JE, Queller DC. 2020 Wild *Dictyostelium discoideum* social amoebae show plastic responses to the presence of nonrelatives during multicellular development. *Ecol. Evol.* **10**, 1119–1134. (doi:10.1002/ece3.5924)
44. Noh S, Geist KS, Tian X, Strassmann JE, Queller DC. 2018 Genetic signatures of microbial altruism and cheating in social amoebas in the wild. *Proc. Natl Acad. Sci. USA* **115**, 3096–3101. (doi:10.1073/pnas.1720324115)
45. de Oliveira JL *et al.* 2019 Conditional expression explains molecular evolution of social genes in a microbe. *Nat. Commun.* **10**, 1–12. (doi:10.1038/s41467-019-11237-2)
46. Tarnita CE, Washburne A, Martinez-Garcia R, Sgro AE, Levin SA. 2015 Fitness tradeoffs between spores and nonaggregating cells can explain the coexistence of diverse genotypes in cellular slime molds. *Proc. Natl Acad. Sci. USA* **112**, 2776–2781. (doi:10.1073/pnas.1424242112)
47. Martinez-Garcia R, Tarnita CE. 2016 Lack of ecological and life history context can create the illusion of social interactions in *Dictyostelium discoideum*. *PLoS Comput. Biol.* **12**, e1005246. (doi:10.1371/journal.pcbi.1005246)
48. Wolf JB, Howie JA, Parkinson K, Gruenheit N, Melo D, Rozen D, Thompson CRL. 2015 Fitness trade-offs result in the illusion of social success. *Curr. Biol.* **25**, 1086–1090. (doi:10.1016/j.cub.2015.02.061)
49. Redfield RJ. 2002 Is quorum sensing a side effect of diffusion sensing? *Trends Microbiol.* **10**, 365–370. (doi:10.1016/S0966-842X(02)02400-9)
50. Jefferson KK. 2004 What drives bacteria to produce a biofilm? *FEMS Microbiol. Lett.* **236**, 163–173. (doi:10.1111/j.1574-6968.2004.tb09643.x)
51. Lahti DC, Johnson NA, Ajie BC, Otto SP, Hendry AP, Blumstein DT, Coss RG, Donohue K, Foster SA. 2009 Relaxed selection in the wild. *Trends Ecol. Evol.* **24**, 487–496. (doi:10.1016/j.tree.2009.03.010)
52. Johnson NA, Lahti DC, Blumstein DT. 2012 Combating the assumption of evolutionary progress: lessons from the decay and loss of traits. *Evol. Educ. Outreach* **5**, 128–138. (doi:10.1007/s12052-011-0381-y)
53. Sadier A, Sears KE, Womack M. 2022 Unraveling the heritage of lost traits. *J. Exp. Zool. B Mol. Dev. Evol.* **338**, 107–118. (doi:10.1002/jez.b.23030)
54. Morris JJ, Lenski RE, Zinser ER. 2012 The Black Queen Hypothesis: evolution of dependencies through adaptive gene loss. *MBio* **3**, e00036-12. (doi:10.1128/mBio.00036-12)
55. Castillo DI, Switz GT, Foster KR, Queller DC, Strassmann JE. 2005 A cost to chimerism in *Dictyostelium discoideum* on natural substrates. *Evol. Ecol. Res.* **7**, 263–271.
56. Loomis WF, Sussman M. 1966 Commitment to the synthesis of a specific enzyme during cellular slime mold development. *J. Mol. Biol.* **22**, 401. (doi:10.1016/0022-2836(66)90148-3)
57. DiSalvo S, Haselkorn TS, Bashir U, Jimenez D, Brock DA, Queller DC, Strassmann JE. 2015 Burkholderia bacteria infectiously induce the proto-farming symbiosis of *Dictyostelium* amoebae and food bacteria. *Proc. Natl Acad. Sci. USA* **112**, E5029–E5037. (doi:10.1073/pnas.1511878112)
58. Brock DA, Douglas TE, Queller DC, Strassmann JE. 2011 Primitive agriculture in a social amoeba. *Nature* **469**, 393–396. (doi:10.1038/nature09668)
59. Garcia JR, Larsen TJ, Queller DC, Strassmann JE. 2019 Fitness costs and benefits vary for two facultative Burkholderia symbionts of the social amoeba, *Dictyostelium discoideum*. *Ecol. Evol.* **9**, 9878–9890. (doi:10.1002/ece3.5529)
60. Smith MH. 2004 *Genotypic diversity and population structure in dictyostelium discoideum*. Houston, TX: Rice University.
61. Schindelin J *et al.* 2012 Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682. (doi:10.1038/nmeth.2019)
62. R Core Team. 2015. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. See <https://www.R-project.org>.
63. Bates *et al.* 2015. Package 'lme4'. See <http://lme4.r-forge.r-project.org>.
64. Lenth 2022. Package 'emmeans'. See <https://cran.r-project.org/web/packages/emmeans/index.html>.
65. Berner D, Amrhein V. 2022 Why and how we should join the shift from significance testing to estimation. *J. Evol. Biol.* **35**, 777–787. (doi:10.1111/jeb.14009)
66. Darwin C. 1871 *The descent of man: and selection in relation to sex*. London, UK: John Murray.
67. Darwin C. 1859 *On the origin of species by means of natural selection*. London, UK: J. Murray.
68. McCutcheon JP, Moran NA. 2012 Extreme genome reduction in symbiotic bacteria. *Nat. Rev. Microbiol.* **10**, 13–26. (doi:10.1038/nrmicro2670)
69. Smith EE *et al.* 2006 Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc. Natl Acad. Sci. USA* **103**, 8487–8492. (doi:10.1073/pnas.0602138103)
70. Wcislo WT, Danforth BN. 1997 Secondly solitary: the evolutionary loss of social behavior. *Trends Ecol. Evol.* **12**, 468–474. (doi:10.1016/S0169-5347(97)01198-1)
71. Badgett MR, Auer A, Carmichael LE, Parrish CR, Bull JJ. 2002 Evolutionary dynamics of viral attenuation. *J. Virol.* **76**, 10 524–10 529. (doi:10.1128/JVI.76.20.10524-10529.2002)
72. Carthey AJ, Banks PB. 2014 Naïveté in novel ecological interactions: lessons from theory and

- experimental evidence. *Biol. Rev.* **89**, 932–949. (doi:10.1111/brv.12087)
73. Berger S, Wikelski M, Romero LM, Kalko EK, Rödl T. 2007 Behavioral and physiological adjustments to new predators in an endemic island species, the Galápagos marine iguana. *Horm. Behav.* **52**, 653–663. (doi:10.1016/j.yhbeh.2007.08.004)
74. Atkinson I. 2006 Introduced mammals in a new environment. In *Biological invasions in New Zealand*, pp. 49–66. Berlin, Germany: Springer.
75. Lee M-C, Marx CJ. 2012 Repeated, selection-driven genome reduction of accessory genes in experimental populations. *PLoS Genet.* **8**, e1002651. (doi:10.1371/journal.pgen.1002651)
76. Renda BA, Dasgupta A, Leon D, Barrick JE. 2015 Genome instability mediates the loss of key traits by *Acinetobacter baylyi* ADP1 during laboratory evolution. *J. Bacteriol.* **197**, 872–881. (doi:10.1128/JB.02263-14)
77. Hoffmann AA, Hallas R, Sinclair C, Partridge L. 2001 Rapid loss of stress resistance in *Drosophila melanogaster* under adaptation to laboratory culture. *Evolution* **55**, 436–438.
78. Holland B, Rice WR. 1999 Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proc. Natl Acad. Sci. USA* **96**, 5083–5088. (doi:10.1073/pnas.96.9.5083)
79. Jaenike J. 1993 Rapid evolution of host specificity in a parasitic nematode. *Evol. Ecol.* **7**, 103–108. (doi:10.1007/BF01237736)
80. Velicer GJ, Kroos L, Lenski RE. 1998 Loss of social behaviors by *Myxococcus xanthus* during evolution in an unstructured habitat. *Proc. Natl Acad. Sci. USA* **95**, 12 376–12 380. (doi:10.1073/pnas.95.21.12376)
81. Behe MJ. 2010 Experimental evolution, loss-of-function mutations, and ‘the first rule of adaptive evolution’. *Q. Rev. Biol.* **85**, 419–445. (doi:10.1086/656902)
82. Nilsson AI, Koskiniemi S, Eriksson S, Kugelberg E, Hinton JCD, Andersson DI. 2005 Bacterial genome size reduction by experimental evolution. *Proc. Natl Acad. Sci. USA* **102**, 12 112–12 116. (doi:10.1073/pnas.0503654102)
83. Foster KR, Fortunato A, Strassmann JE, Queller DC. 2002 The costs and benefits of being a chimera. *Proc. R. Soc. Lond. B* **269**, 2357–2362. (doi:10.1098/rspb.2002.2163)
84. Ostrowski EA, Rozen DE, Lenski RE. 2005 Pleiotropic effects of beneficial mutations in *Escherichia coli*. *Evolution* **59**, 2343–2352.
85. Khan AI, Dinh DM, Schneider D, Lenski RE, Cooper TF. 2011 Negative epistasis between beneficial mutations in an evolving bacterial population. *Science* **332**, 1193–1196. (doi:10.1126/science.1203801)
86. Cooper TF, Ostrowski EA, Travisano M. 2007 A negative relationship between mutation pleiotropy and fitness effect in yeast. *Evol. Int. J. Organ. Evol.* **61**, 1495–1499. (doi:10.1111/j.1558-5646.2007.00109.x)
87. Fisher RA. 1930 *The genetical theory of natural selection*, p. xiv, 272. Oxford, UK: Clarendon Press.
88. Chen P, Zhang J. 2020 Antagonistic pleiotropy conceals molecular adaptations in changing environments. *Nat. Ecol. Evol.* **4**, 461–469. (doi:10.1038/s41559-020-1107-8)
89. Zhang J. 2023 Patterns and evolutionary consequences of pleiotropy. *Ann. Rev. Ecol. Evol. Syst.* **54**, 1–19. (doi:10.1146/annurev-ecolsys-022323-083451)
90. Hill WG, Zhang X-S. 2012 Assessing pleiotropy and its evolutionary consequences: pleiotropy is not necessarily limited, nor need it hinder the evolution of complexity. *Nat. Rev. Genet.* **13**, 296. (doi:10.1038/nrg2949-c1)
91. Wagner GP, Zhang J. 2011 The pleiotropic structure of the genotype–phenotype map: the evolvability of complex organisms. *Nat. Rev. Genet.* **12**, 204–213. (doi:10.1038/nrg2949)
92. Leroi AM *et al.* 2005 What evidence is there for the existence of individual genes with antagonistic pleiotropic effects? *Mech. Ageing Dev.* **126**, 421–429. (doi:10.1016/j.mad.2004.07.012)
93. Williams PD, Day T. 2003 Antagonistic pleiotropy, mortality source interactions, and the evolutionary theory of senescence. *Evolution* **57**, 1478–1488.
94. Stearns SC. 1989 Trade-offs in life-history evolution. *Funct. Ecol.* **3**, 259–268. (doi:10.2307/2389364)
95. Byars SG, Voskarides K. 2020 Antagonistic pleiotropy in human disease. *J. Mol. Evol.* **88**, 12–25. (doi:10.1007/s00239-019-09923-2)
96. Carter AJ, Nguyen AQ. 2011 Antagonistic pleiotropy as a widespread mechanism for the maintenance of polymorphic disease alleles. *BMC Med. Genet.* **12**, 1–13. (doi:10.1186/1471-2350-12-160)
97. Larsen T. 2023 Experimental evolution of *Dictyostelium discoideum* cheating under relaxed selection. Dryad Digital Repository. (doi:10.5061/dryad.kkwh70s97)
98. Larsen TJ, Jahan I, Brock DA, Strassmann JE, Queller DC. 2023 Reduced social function in experimentally evolved *Dictyostelium discoideum* implies selection for social conflict in nature. Figshare. (doi:10.6084/m9.figshare.c.6961023)