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Does high relatedness promote cheater-free multicellularity in synthetic lifecycles?

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Running title: Evolution of multicellularity

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ABSTRACT

       The evolution of multicellularity is one of the key transitions in evolution and requires extreme levels of cooperation between cells. However, even when cells are genetically identical, non-cooperative cheating mutants can arise that cause a breakdown in cooperation. How then, do multicellular organisms maintain cooperation between cells? A number of mechanisms that increase relatedness amongst cooperative cells have been implicated in the maintenance of cooperative multicellularity including single cell bottlenecks and kin recognition. In this study we explore how relatively simple biological processes such as growth and dispersal can act to increase relatedness and promote multicellular cooperation. Using experimental populations of pseudo-organisms, we found that manipulating growth and dispersal of clones of a social amoeba to create high levels of relatedness was sufficient to prevent the spread of cheating mutants. By contrast cheaters were able to spread under low relatedness conditions. Most surprisingly, we saw the largest increase in cheating mutants under an

experimental treatment that should create intermediate levels of relatedness. This is because one of the factors raising relatedness, structured growth, also causes high vulnerability to growth rate cheaters.

KEY WORDS: single cell bottleneck, *Dictyostelium discoideum*, pseudo-organism, microbial evolution, structured growth, structured dispersal

INTRODUCTION

The appearance of multicellular life over 3 billion years ago represents one of the major innovations in evolutionary history (Maynard Smith & Szathmáry, 1995). However, because multicellularity requires extreme levels of cooperation between cells, multicellular organisms are often vulnerable to the emergence and spread of selfish, non-cooperative ('cheating') cells (Buss, 1987; Michod, 1996; Queller, 2000; Grosberg & Strathmann, 2007). These cheaters may eventually lead to a breakdown in cooperation, which can result in the loss of viable multicellular organisms. In this respect, diseases such as cancer, can be thought of as a form of social cheating (Crespi & Summers, 2005; Aktipis *et al.*, 2015). Cancerous cells promote their own survival at a cost to all cells in the multicellular organism by eventually causing death. Kin selection, the theory that individually costly behaviors can evolve that promote the survival of closely related individuals, has been extremely useful for explaining other evolutionary transitions such as the appearance eusociality in insects (Queller, 2000; Ratnieks *et al.*, 2006). It also suggests that the breakdown of multicellularity can be precluded when relatedness between cooperating cells remains high (Strassmann & Queller, 2007). Previous work has shown that mechanisms such as single cell bottlenecks maybe critical in stabilizing

multicellular cooperation because they create genetically identical cell lineages, thereby limiting the scope for cheating, and may help explain why single cell bottlenecks are present most multicellular lifecycles (Maynard Smith & Szathmáry, 1995; Grosberg & Strathmann, 1998; Roze & Michod, 2001; Grosberg & Strathmann, 2007; Kuzdzal-Fick *et al.*, 2011).

However, not all multicellular organisms undergo single cell bottlenecks (or undergo them only very rarely), instead reproducing through multicellular propagules, such as tubers and stolons in plants or hyphae in fungi (Grosberg & Strathmann, 2007). Conflicts among cell types (or at least the remnants of these conflicts) may still be present in multicellular organisms (Buss, 1987). Therefore, other factors that increase relatedness and reduce conflict among cells may be equally important for the emergence and maintenance of cooperative multicellularity and for formation of an organism more generally (Queller & Strassmann, 2009; 2013; Libby *et al.*, 2016). There are a number of potential mechanisms that can increase relatedness such as common lifecycle events. These include dispersal and growth and are likely to be important in a variety of organisms (Hamilton, 1964; Queller, 1994; Michod, 1996; Kummerli *et al.*, 2009; Buttery *et al.*, 2012; Queller & Strassmann, 2013).

For example, when dispersal to the next generation is limited to only a small, local fraction of cells, this should in principle reduce the levels of genetic variation present in the next generation (i.e. it increases relatedness). This type of dispersal resembles a less extreme form of single cell bottlenecking and can be thought of as a relatively 'structured' form of dispersal where only a few, spatially clustered cells are transferred together. Natural examples of structured dispersal are fairly common and include reproductive strategies such as budding dispersal, where small groups of closely related individuals disperse together and can be found in a range of organisms

from microbes (Pfeiffer & Bonhoeffer, 2003; Kummerli *et al.*, 2009) to cooperative breeding birds (Sharp *et al.*, 2008).

Similarly, growth can also act as a mechanism to limit genetic variation through drift and founder effects within an expanding population (Hallatschek *et al.*, 2007; Buttery *et al.*, 2012; Gralka *et al.*, 2016). When growth is structured and cells give rise to daughter cells that are always close to their relatives, this should on average prevent local interactions between genetic variants. Cheating mutants that arise will tend to interact more often with themselves, thereby limiting their potential to spread. Again, this is relatively common in many multicellular organisms, because cells tend to give rise to cells next to them and movement of individual cells within the organism is limited. However, when there is some movement of cells within an organism, germlines tend to be sequestered to prevent the possibility of cheating mutants spreading to subsequent generations (Buss, 1987).

Although both dispersal and growth can act to increase relatedness and limit the spread of cheaters, it is unclear whether they are sufficient in and of themselves to prevent the spread of cheating mutants. In part, this is due to the difficulty of measuring these effects in multicellular organisms over multiple generations. However, by utilizing multicellular pseudo-organisms composed of genetically identical single-celled microorganisms, it is possible to construct a variety of synthetic life cycles by manipulating how microbes are transferred to new nutrients (dispersal) and by varying the structure of their growth (Queller & Strassmann, 2013). Forcing microbes to proliferate under these different populations structures that mimic features of multicellular growth can allow one to clearly identify the important factors that maintain cooperation in larger, more complex multicellular organisms.

In this study we used the social amoeba *Dictyostelium discoideum* to create multiple pseudo-organisms in order to test how different types of dispersal and growth affect the evolution of multicellularity. This social amoeba forms multicellular structures when faced with starvation. Under nutrient rich conditions it exists as single, freely-moving amoebae, which feed on bacteria. However, when bacteria are scarce amoebae combine to form a motile, migratory slug. The migration of this slug culminates in the formation a multicellular fruiting body composed of dead stalk cells (20%), which hold aloft viable, spore cells (80%) in a sorus.

We first selected for a strain that exhibited cheating during fruiting body formation. Our intent was to find a cheater that operates once during the life cycle of the pseudo-organism, much like mutations posited to gain an advantage by moving into an animal germline (Buss, 1987). We mixed this cheater with wildtype, manipulated both growth and dispersal, and monitored any increase in the cheater strain. We experimentally manipulated growth by either allowing cells to grow out from a central location (structured growth, predicted high local relatedness) or by mixing them evenly across a petri dish (unstructured growth, predicted low relatedness). Previous experiments have shown that when cells grow from a central location, genetic drift across the expanding growth front creates sectors of high relatedness (Hallatschek *et al.*, 2007; Buttery *et al.*, 2012; Gralka *et al.*, 2016), whereas growing cells out from scattered locations generates lower relatedness (smith *et al.*, 2016) and thus mirrors a scenario where cells are able to move throughout an organism by themselves.

We also manipulated dispersal by transferring cells to new plates, representing offspring organisms, from the same location (structured dispersal, predicted high relatedness) or from multiple, different locations (unstructured dispersal, predicted low relatedness). Transferring cells from a spatially localized area acts as a less stringent

form of germ line sequestration and bottlenecking between generations, while collecting cells from across an organism should reduce overall relatedness in the subsequent generation. Although there is perhaps no analogous life cycle to this latter outcome, it can be instructive to consider why certain lifecycles are not found in nature (Fisher, 1930).

This study is in some respects almost like a computer simulation, where the conclusions are determined directly from the premises and starting conditions, so we expected our predictions to be confirmed. However, because there is real biology in the system, there is the potential for interesting surprises, and we got a mixture of confirmation and surprise. As predicted, we found that combining the two high-relatedness conditions was sufficient to prevent the spread of a mutant that cheats its neighbors during fruiting body formation, whereas combining the two low-relatedness conditions allowed cheaters to spread. Surprisingly, the largest increase of cheaters occurred under conditions that should create intermediate levels of relatedness, where growth was structured but dispersal unstructured. On further investigation, we found that this was because the fruiting body cheating mutant also had a higher growth rate, demonstrating the particular danger of growth rate mutants to multicellularity.

METHODS AND MATERIALS

Isolation of *D. discoideum* Non-fruiters

In order to conduct these evolution experiments, where we monitor the spread of a cheating mutant that is unable to form a multicellular fruiting body, we first needed to isolate a clone that could not form fruiting bodies on its own. We therefore serially passaged a wild *D. discoideum* clone NC28.1 tagged with a chromosomally expressed red fluorescent protein (RFP) under low relatedness conditions that have previously

been shown to favor non-fruiting cheating mutants (Kuzdzal-Fick *et al.*, 2011; Buttery *et al.*, 2012). It was grown in the presence of 100ul of *Klebsiella pneumoniae,* diluted to an O.D.$_{600nm}$ of 1.5, as a food source on SM/5 agar plates (2g peptone, 0.2g yeast extract, 2g glucose, 1.9g KH_2PO_4, 1g K_2HPO_4, 0.01g $MgSO_4$ g, and 15g agar per liter) at room temperature for 1 week. After 4 days of growth, we collected sorus contents from the entire plate were and diluted to it 10^7 cells/ml. We then transferred 100ul of spore cells (10^6 cells in total) to fresh SM/5 agar plates with *K. pneumoniae*. This process was repeated a further 14 times (15 transfers in total) for 12 replicate lines.

After the final transfer, we collected the sorus contents from each of the 12 plates and diluted to 50 cells/ml. 100ul (~5 cells in total) were plated on 120 SM/5 plates with *K. pneumoniae* (10 plates for each evolved line). We incubated plates for 48 hours at room temperature and screened for amoeba formed plaques. We harvested amoebae from all detectable plaques and transferred them to fresh SM/5 agar plates with *K. pneumoniae*. These were incubated at room temperature for 1 week. We screened these plates for the presence of fruiting bodies. We harvested cells from plates that failed to form fruiting bodies and stored them in HL5 (5g proteose peptone, 5g thiotone E peptone, 10g glucose, 5g yeast extract, 0.35 g $Na_2HPO_4 * 7H_2O$, 0.35g KH_2PO_4 per liter) with 10% DMSO at -80C.

We tested the *D. discoideum* clones that did not form fruiting bodies (9 in total), to see whether they are social cheaters as reported in other studies (i.e. they are overrepresented in the sorus compared their initial starting frequency when competed with the non-evolved, ancestral wildtype clone)(Ennis *et al.*, 2000; Kuzdzal-Fick *et al.*, 2011). These non-fruiters were revived from frozen stocks and competed with the ancestral wildtype. We harvested amoeba cells from each strain and washed them twice in KK2 buffer (2.2g KH_2PO_4 and 0.7g K_2HPO_4 per liter). We placed 10^5 cells of a 9:1 ratio

of ancestral to evolved cells on sterile filter paper with 2ml KK2. We harvested sorus contents after 48 hours of incubation and measured the relative frequency of RFP-tagged spore cells (i.e. our putative cheating strains) using a flow cytometer (BD Accuri C6).

Selection Experiment

In this selection experiment we tracked the changes in the frequency of an untagged, ancestral strain compared to a RFP-tagged, evolved strain. Mixtures of these two strains where grown on a total of 24 agar plates, with each plate representing a pseudo-organism. We varied both growth structure and the mode of dispersal, in a fully factorial design, to create 4 conditions (i.e. 6 plates/ pseudo-organisms for each condition): structured growth and structured dispersal (predicted high relatedness), structured growth and unstructured dispersal (predicted intermediate relatedness), unstructured growth and structured dispersal (predicted intermediated relatedness), unstructured growth and unstructured dispersal (predicted low relatedness) (Fig. 1).

Amoeba cells of NC28.1 (ancestral strain) and evolved non-fruiting cheater clone 2 (EC28.2) were first harvested, diluted, and mixed to 10^6 cells/ml, with 99:1 ratio of NC28.1 and EC28.2. In order to create structured growth, we pipetted 100ul (10^5 cells in total) of this mixture onto the center of SM/5 agar plates with *K. pneumoniae* to allow cells to grow out from a central location. This should create sectors of high relatedness and allow cooperating cells to interact with each other. Alternatively we spread this mixture evenly across plates in order to create unstructured growth (Fig. 1). This should increase the interaction between cheaters (EC28.2) and cooperators (NC28.1) and lower relatedness. We incubated the plates at room temperature for 1 week. After incubation, we collected 16 sori from each plate. In order to create unstructured

dispersal, we collected from 16 separate locations along a circular transect with a radius of 3 centimeters. In order to create structured dispersal, we collected 16 sori from a single randomly chosen location on this circular transect (as illustrated in Figs. 1 and S1). We counted spore cells and diluted them to 10^6 cells/ml and 100ul was transferred to fresh SM/5 plates with *K. pneumoniae*, by either pipetting in the center or spreading across plates. This process was repeated a further 3 times, for a total of 4 transfers and 5 growth cycles. After the final round of growth, we randomly collected 16 sori from each plate and diluted each in 100ul *K. pneumoniae*. Sorus contents were individually analyzed using flow cytometry.

To test for differences between our experimental treatments we performed a linear mixed effects model where individually measured sori frequencies were nested within replicate pseudo-organisms (percent of non-fruiters ~ experimental treatments, random effect = individual sori frequencies nested within each pseudo-organism). P-values were obtained by comparing the full model against a null model (without the treatment effect) using a likelihood ratio test. In order to test whether pseudo-organisms where cheaters vanished completely (due to drift) affected our analysis, we re-ran the same analysis with all zero values removed and found no qualitative difference.

Measuring Expansion and Growth Rate

In order to test whether the non-fruiting cheater mutant EC28.2 is able to expand across a nutrient agar plate faster than its ancestral NC28.1, we pipetted 100ul of 10^5 amoeba cells of each strain onto the center of SM/5 agar plates with *K. pneumoniae*. After 16 hours of growth the zone of bacterial clearance caused by feeding amoeba cells was recorded and measured along three random transects starting from

the center of the plate. These same plates were then reassessed every 8 hours and zones of clearance were again measured. This was performed on 3 plates of each strain (6 plates in total), until a total of 48 hours had elapsed. In order to test for differences in expansion rate, the distance travelled by amoebae (as measured by zones of clearance) was averaged across the three transects for each plate at each time point. An Analysis of Covariance (ANCOVA) was performed on this data (ln(distance travelled ~ time*strain)), using the interaction term time*strain to determine whether there was a significant difference between the expansion rate of both strains.

To see whether this increase in expansion rate is due to faster vegetative growth, we measured the growth rate of both strains, by spreading 10^4 log-phase cells of each strain separately on SM/5 agar plates with *K. pneumoniae*. After incubating for 18 hours, cells were collected from the plate, diluted in KK2, and each sample was counted three times using a haemacytometer. We assayed plates every 4 hours, measuring growth on 3 separate plates of each strain at every time point (a total of 30 plates), until 34 hours of growth had elapsed. To test for differences in growth rates cell counts were averaged for each plate and an ANCOVA was performed on the natural-log transformed cell growth data (ln(cell counts) ~ time*strain), using the interaction term time*strain to determine whether there was a significant difference between the growth of the strains.

Finally, to confirm that an increase in growth rate and expansion translates into a competitive advantage for EC28.2 over NC28.1 in our structured growth experimental system, we competed both strains at a 9:1 ratio of NC28.1 and EC28.2 by pipetting a mixture of both onto the center of SM/5 agar plates with *K. pneumoniae*. These plates were incubated at room temperature for 1 week, and sori were collected at centimeter intervals, from 3 randomly drawn transects originating from the center of the plate. Sorus contents were individually counted using flow cytometry. In order to determine

whether EC28.2 increases in frequency as it grows across a nutrient agar plate we took the average of the three transects per plate and performed a linear regression on our arcsine transformed proportion data (proportion of EC28.2~distance).

Analysis

All flow cytometry data were analyzed using Bioconductor (Gentleman *et al.*, 2004) as previously described (smith *et al.*, 2016). Statistics as described in the text were performed in R version 3.2.3 (R Core Team, 2015).

RESULTS

After evolving NC28.1 for 15 growth cycles, about 180 generations, under conditions that select for the evolution of non-fruiting cheaters (i.e. low-relatedness), we were able to isolate 9 non-fruiting clones. Of these clones only two also acted as social cheaters and were overrepresented in the sorus contents of fruiting bodies (Fig 2). Evolved clone 2 (EC28.2) was chosen as a non-fruiting cheater for our evolution experiments, because it exhibited the largest increase in the sori of fruiting bodies.

We then created multicellular pseudo-organisms by seeding a mixture of 99% NC28.1 and 1% EC28.2 on agar petri dishes. These pseudo-organisms underwent 5 cycles of growth and 4 dispersal events. Growth and dispersal were manipulated to create conditions where pseudo-organisms experienced structured or unstructured growth and structured or unstructured dispersal in a fully factorial experimental design (as seen in Fig 1).

There was a strong effect of varying levels of growth and dispersal ($\chi^2(3)$ = 1150.5, p < 0.001), with non-fruiting, cheating cells (EC28.2) increasing in frequency in four of the six replicate pseudo-organisms under conditions of unstructured growth and

unstructured dispersal (Fig 3). Conversely, non-fruiting cheaters spread less under conditions of structured growth and structured dispersal and conditions of unstructured growth and structured dispersal. The largest increase in non-fruiting cheaters was seen in pseudo-organisms that underwent cycles of structured growth and unstructured dispersal events, where non-fruiting cheaters increased in frequency in all pseudo-organisms. This is somewhat surprising as previous studies have shown that structured growth increases relatedness, which should in principle limit the spread of cheaters (Buttery *et al.*, 2012).

One possible explanation for these results is that non-fruiting cheaters are also better able to spread across the petri dish, thereby outcompeting cooperative fruiting cells during the growth phase as well as during fruiting body formation. Structured growth from the center allows for more generations of competition than unstructured growth from numerous initial starting points. Microbial growth in range expansions provides larger fitness benefits compared to uniformly expanding microbial populations (Gralka *et al.*, 2016). In order to test this prediction, we measured the expansion rates of both strains (NC28.1 and EC28.2) by pipetting each strain separately in the center of a petri dish and measuring the speed at which they grow out across the plate (similar to our structured growth evolution experiment). We found that EC28.2 was indeed able to expand across the petri dish faster (ANCOVA: $F_{1,27}$ = 10.26, p < 0.004 for the interaction term time*strain)(Fig 4). We then confirmed that EC28.2's increased expansion rate is in part due to an increase in growth rate by evenly spreading each strain over a petri dish (similar to our unstructured growth evolution experiment) and measuring amoeba density at multiple time points (ANCOVA on the log-transformed amoeba density: $F_{1,27}$ = 23.93, p < 0.001 for the interaction term time*strain)(Fig 5).

To further confirm that this increase in growth and expansion results in EC28.2 outcompeting NC28.1 under our experimental setup, we performed an additional experiment where we pipetted NC28.1 and EC28.2 on the center of a petri dish and allowed them to grow outwards. We measured the respective frequencies of both strains along multiple transects starting at the center of the plate, and again, we found that EC28.2 showed much higher frequencies at the edge of the plate than the center (linear regression, $F_{1,8}$ = 12.75, p < 0.01)(Fig. 6).

DISCUSSION

In this study we tested how varying levels of structured growth and dispersal affect the evolutionary stability of multicellularity. Using pseudo-organisms composed of the social amoeba, *D. discoideum*, we manipulated both growth, by either spreading cells evenly or allowing them to grow out from the center of the plate, and dispersal, by either transferring several spores from one location or one spore from several locations. This allowed us to create relatively high and low levels of structure in growth and dispersal during the lifecycles of our pseudo-organisms. We then tracked the spread of an RFP-labeled, non-fruiting mutant in our pseudo-organisms undergoing varying levels of structured growth and dispersal.

As expected, we found that non-fruiting cheaters were unable to spread when both growth and dispersal were structured. This occurs because allowing cells to grow out from the center of the plate creates sectors of high relatedness (Buttery *et al.*, 2012) and allowing cells to disperse only from one highly related sector purges the pseudo-organism of non-fruiting cheaters. In this respect, high levels of structured growth and dispersal act in similar manners to germline sequestration and single cell bottlenecks, respectively, but on a lesser scale (Buss, 1987; Kuzdzal-Fick *et al.*, 2011).

We also found that under relatively low levels of structure in both growth and dispersal, non-fruiting cheaters were able to invade (Kuzdzal-Fick *et al.*, 2011). This not surprising because these conditions create low levels of relatedness where non-fruiting cheaters are able to interact with and easily outcompete fruiting body forming cells by increasing their frequency in the sorus compared to wildtype spores.

More intriguingly, we saw starkly different outcomes in the two treatments that combined different levels of structure in either of the two treatments (i.e. growth and dispersal), which we assumed would generate intermediate relatedness and therefore intermediate protection against the non-fruiting cheat. During unstructured growth but structured dispersal we observed that non-fruiting cheaters were unable to invade, suggesting that in this system structured dispersal is sufficient to preclude the emergence and spread of cheaters. This finding is in line with previous observations, which have shown that fine-scale spatial structure can create high levels of relatedness (smith *et al.*, 2016). As sorus contents were transferred from spatially clustered fruiting bodies and the initial frequency of cheaters was rare, this fine-scale patterning presumably allowed pseudo-organisms to eliminate the cheating cell line. Overall, it is a striking pattern that our two structured dispersal treatments controlled cheating effectively, regardless of the growth structure, and our two unstructured dispersal treatments allowed cheaters to increase. This seems to be in accord with the fact that structured dispersal, often in the form of a single-cell bottleneck, is extremely common in nature.

Interestingly, we saw by far the largest increase of non-fruiting cheaters in pseudo-organisms that underwent high levels of structured growth but relatively unstructured dispersal. This may at first seem surprising because structured growth creates sectors of high relatedness when *D. discoideum* is grown on plates (Buttery *et al.*,

2012). However, the non-fruiting mutant we competed against our ancestral cooperator was not only a social cheat but also exhibited a slight increase in growth rate. This growth advantage allows the mutant to outcompete the ancestral wildtype strain as the amoebae replicate across the plate, increasing the relative frequency of the cheaters as it expands from the origin of growth. This also explains why more non-fruiting cheaters were present compared to pseudo-organisms that underwent both unstructured growth and dispersal. When growth-rate cheaters grow from the center of a plate, they experience more generations to benefit from their growth rate advantage, compared to when spread evenly. This modest growth rate advantage (Figs. 4 and 5) translates into a much larger competitive advantage (Fig. 6) because the growth rate mutants are faster at colonizing new areas of the plate, thereby reducing the area and resources available for wildtype amoeba. In this respect, our system acts similarly to previously reported results of range expansions in budding yeast, where mutants display larger fitness increases during range expansions than during uniform population expansions (Gralka *et al.*, 2016).

However, this alone does not explain why there was no similar increase in cheaters in the other treatment with structured growth. This other treatment differed only in having structured dispersal. It therefore seems that structured dispersal selects against cheating but it is not obvious why this is so. Both structured growth treatments should result in sectors of mostly fruiting cooperators and sectors of cheaters with far fewer fruiting bodies. It could be that in choosing 16 individual fruiting bodies from 16 locations we could always find one decent fruiting body even in bad sectors while if we sampled 16 from one bad location we would have to move into a cooperator sector to get sufficient fruiting bodies, effectively selecting against cheaters. Alternatively, since structured dispersal essentially samples only one sector and we started with only 1%

cheaters, we may simply have failed by chance to sample cheater sectors. Starting with higher percentage might have led to different results but our intent was to study the invasion of rare cheaters. To explore this more thoroughly we would need a larger sample of pseudo-organisms to average better over the stochastic effects.

We do not know if the two cheating effects, during growth and during fruiting, are the result of a single mutation or more than one. The only non-fruiting cheater mutation that has been characterized in *D. discoideum*, called *chtA-* or *fbxA-*, did not show a pleiotropic effect on growth rate (Ennis et al. 2000). However, cheating mutants that also exhibit increases in growth rate are likely to be relatively common, because many cheating mutations result from a loss of function such as cooperative genes involved exoproduct secretion, behaviors that determine virulence, or the formation of complex multicellular structures (Griffin *et al.*, 2004; Velicer *et al.*, 2006; Diggle *et al.*, 2007; Ackermann *et al.*, 2008; Santorelli *et al.,* 2008). Recent studies have suggested loss of function mutations can provide fitness advantages outside of any social interaction, as cells are better able optimize their metabolism in novel environments and no longer engage in costly biosynthetic pathways (Hottes *et al.*, 2013). Also in other systems, growth rate mutants can themselves be viewed as social cheaters. For example, prudent behaviors such as conserving resources are prone to cheating by individuals that continue to consume and grow (Buckling & Brockhurst, 2008). Perhaps one of the most egregious examples is the evolution of cancer, where uncontrollably proliferating cells gain a short-term fitness advantage to the ultimate detriment of the multicellular host (Crespi & Summers, 2005; Aktipis *et al.*, 2015). In this respect, the non-fruiting cheaters isolated in our study act in an analogous manner to a cancerous disease (in this case a heritable one) by eroding multicellularity through a toxic combination of social cheating and increased growth.

We initially designed our experiment to test for protection against cheaters that gain an advantage during one particular point in development, hence our selection for a cheat of the fruiting body stage. This corresponds to the kinds of germline cheaters envisioned by Buss (Buss, 1987) that do something, at a particular time and place during development, to move into the germline. However, we inadvertently also selected for a growth rate cheater and our unexpected result arose because the structured growth treatment that should decrease fruiting-body cheating by increasing relatedness at the fruiting stage actually creates the best conditions for a growth-rate cheater. Because these cheaters can gain an extended advantage throughout development, they can gain relatively large advantages (Michod, 1996; Queller, 2000). This advantage is compounded when the growth rate cheater is competing in a spatially structured environment, because it is able to gradually block the expansion of wildtype cells. Our experiment ended up being dominated by this growth-rate advantage, and it demonstrated the particular danger of these kinds of cheaters. For multicellular organisms that must regulate growth patterns to achieve adapted morphologies, they must have controls, such as single-cell bottlenecks, to prevent the spread of these cheaters. However, our results do not necessarily mean that structured growth is always impotent against cheaters. Stronger forms of structured growth might limit cheating better. Suppose, for example, we had set up radial barriers on the plate, such that cells from one sector could not move across the barrier into other sectors. This should reduce the lateral spread of growth rate cheaters in a manner similar to branching in plants or filamentous fungi, where cells can't move from one branch to the other.

Our findings suggest that while some mechanisms that increase relatedness (i.e. structured dispersal) can be effective at eradicating cheating cells, others such as

structured growth (under certain conditions) can accelerate the spreading of some

cheaters. This highlights the important role that single cell bottlenecks play in the

evolution of multicellularity and may help explain why only relatively simple forms of

multicellularity exist in organisms such as *D. discoideum* where thousands of cells may

be co-dispersed. However, it is important to note that dispersal and growth are not the

only mechanisms that prevent the emergence and spread of cheaters, and other factors

that increase relatedness, such as kin recognition, may be equally important.

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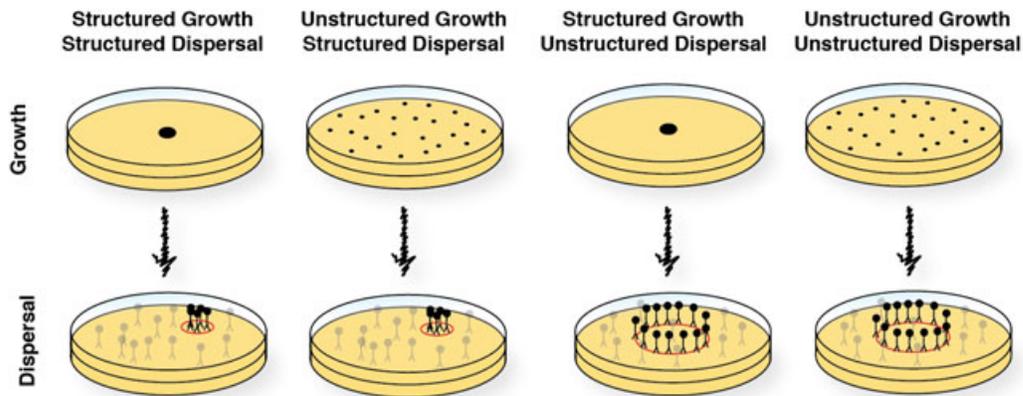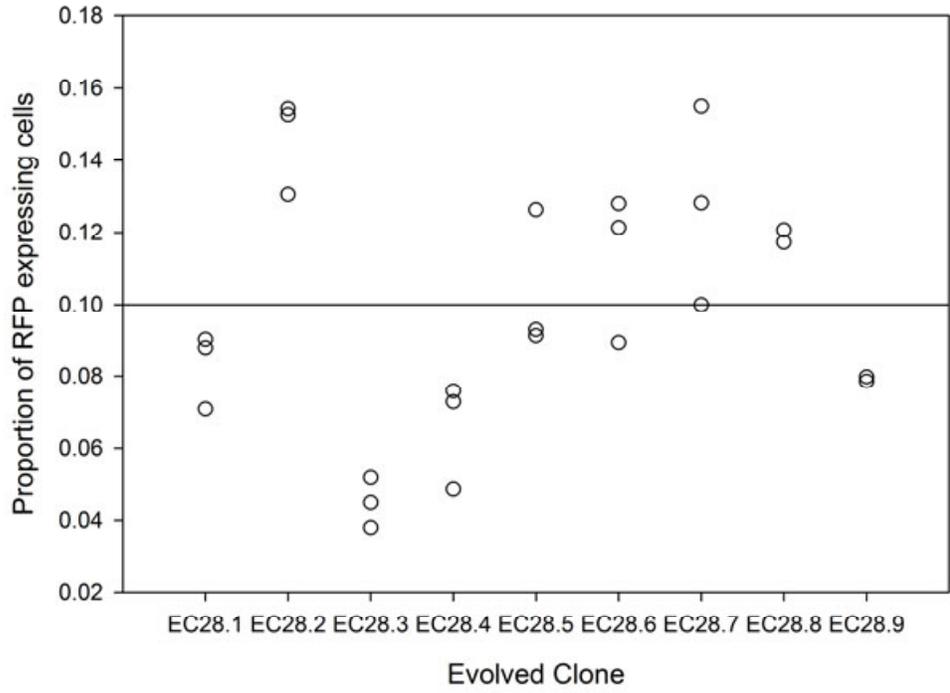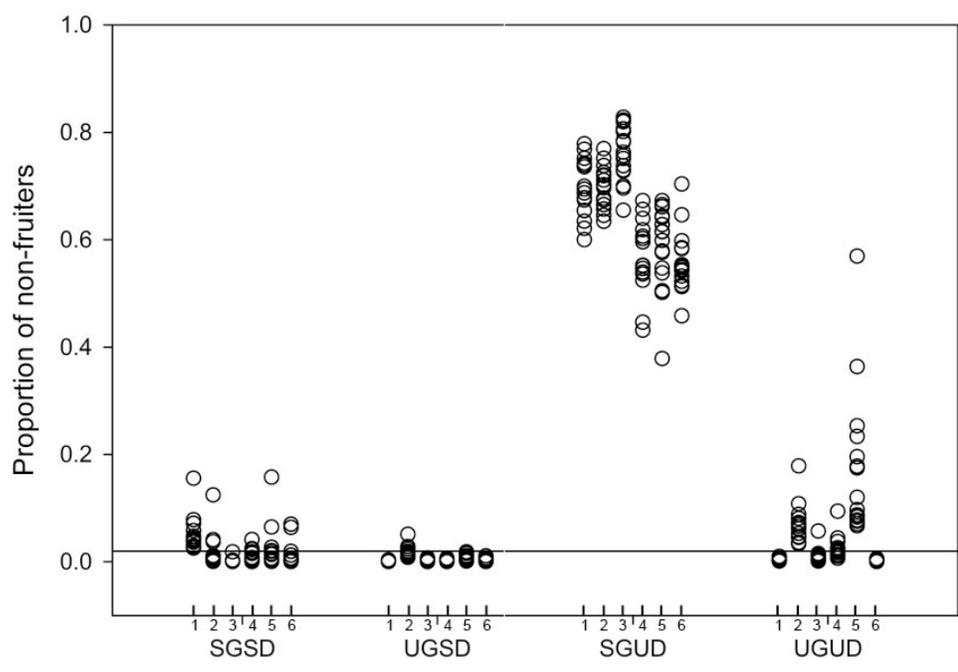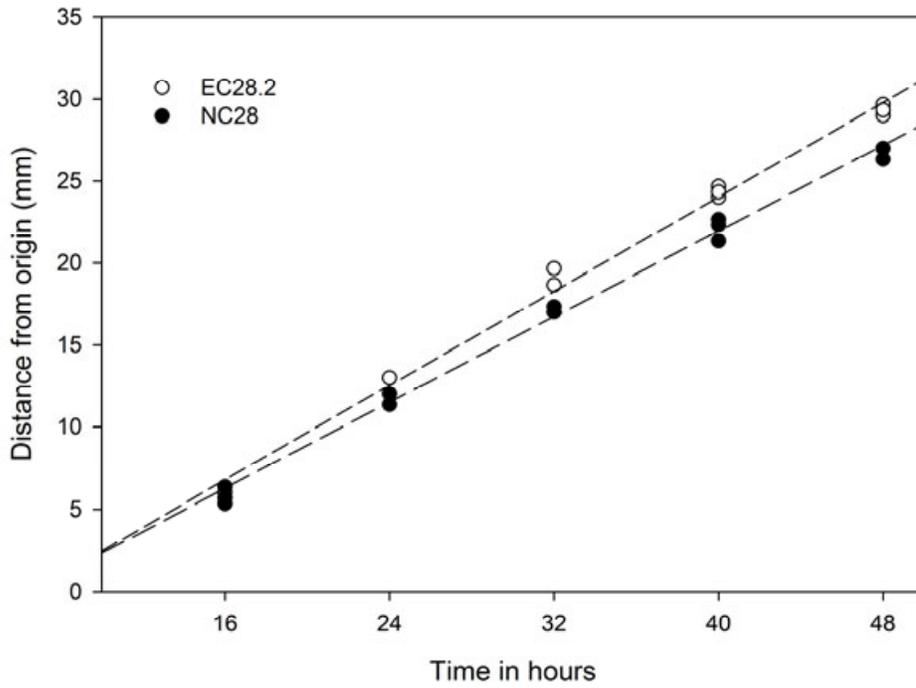
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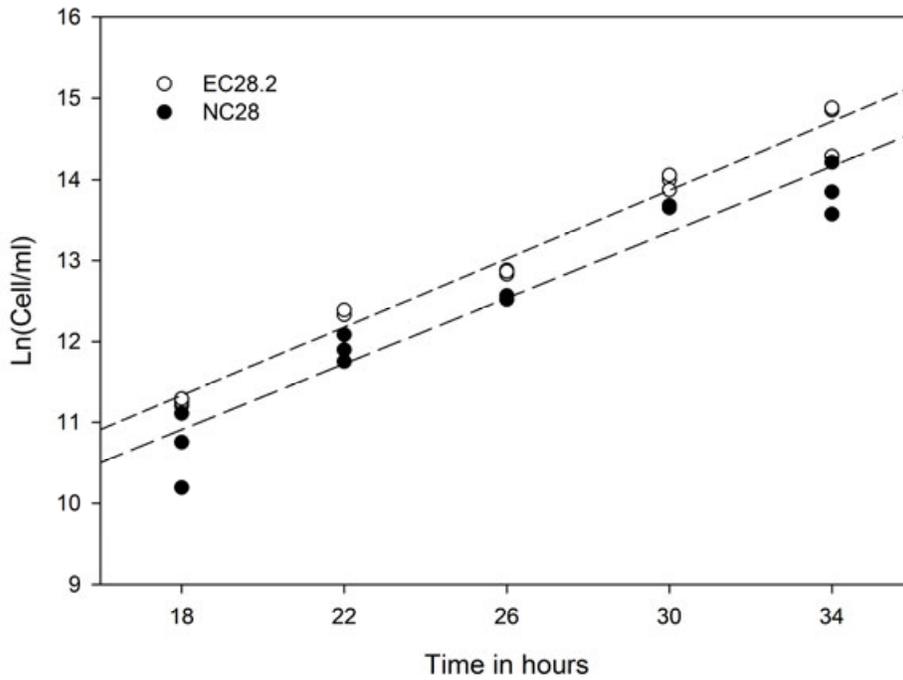
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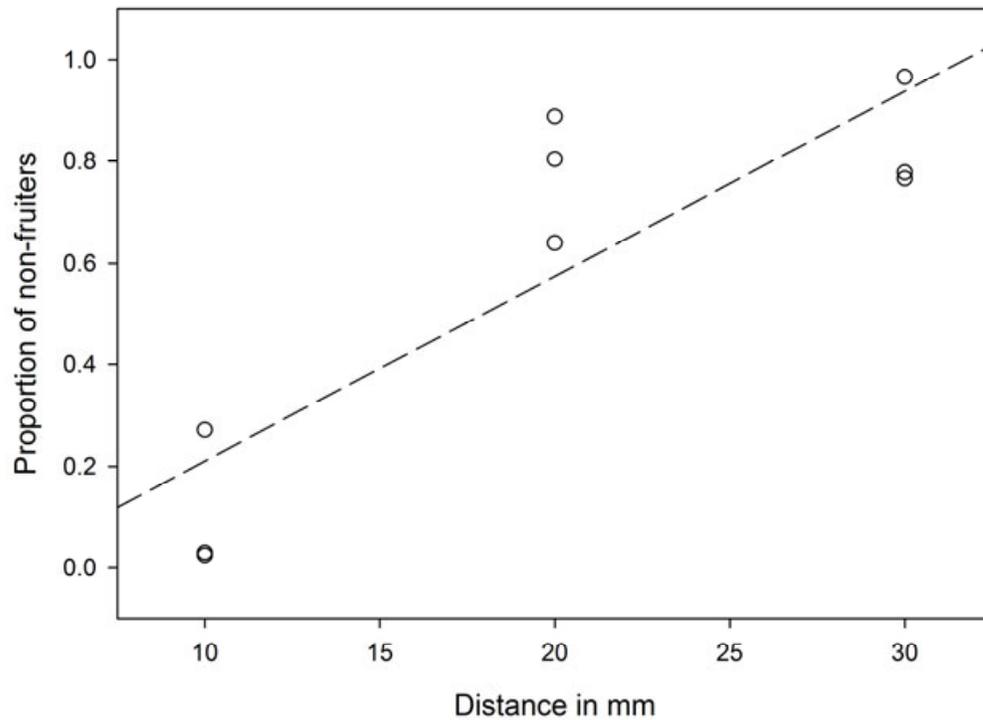

Figure 3

Figure 4

Figure 5

Figure 6

FIGURE LEGENDS

Figure 1. A schematic diagram of how relatedness within pseudo-organisms was experimentally manipulated, by 1) either creating structured vs. unstructured growth by mixing cells or allowing them grow from a central location on the petri dish and 2) creating structured vs. unstructured dispersal by picking sori from across in the plate in a circular transect or from one cluster along that transect.

Figure 2. Replicate plates of NC28.1 were passaged 15 times and 9 non-fruiting body forming plaques were isolated. These 9 evolved non-fruiters were individually competed against the ancestral strain NC28.1 under starvation conditions on filter disks, to prevent growth. Sorus contents were analyzed using flow cytometry and only

two evolved strains acted as social cheaters (EC28.2 and EC28.8) and were over represented in the sorus compared to their starting frequency as amoeba.

Figure 3. EC28.2 was competed against the ancestral strain NC28.1 under a range of different spatial and growth structures. Strain frequencies were measured with flow cytometry for 16 sori (each plotted as a circle) from each of the 6 pseudo-organisms (vertical rows of circles) in each treatment. When both growth and dispersal were highly structured (SGSD), non-fruiting cheaters failed on invade the pseudo-organism as a whole. Non-fruiting cheaters also failed to invade experimental populations under unstructured growth and structured dispersal (UGSD). The largest increase in non-fruiters was seen when growth was structured but dispersal unstructured (SGUD). Finally non-fruiting cheaters were able to invade when both growth and dispersal were both unstructured.

Figure 4. The speed at which the ancestral (NC28.1) and evolved non-fruiting (EC28.2) strains are able expand across a petri dish was measured in order to determine whether other fitness differences exist, other than their ability to form fruiting bodies. EC28.1 was able to expand much faster compared to the ancestral NC28.1. Dashed lines represent linear regressions for both strains.

Figure 5. Growth rates for both strains were measured by spreading each strain evenly across multiple petri dishes and counting the increase in amoebas over time. EC28.1 grows much faster compared to the ancestral NC28.1, which gives it an added fitness advantage. Dashed lines represent polynomial linear regressions for both strains.

Figure 6. EC28.1's small growth rate advantage translates in a large competitive advantage as it grows out across the petri dish when starting at a frequency of 10%. It easily outcompetes NC28.1 and reaches higher frequencies the further it travels from the origin of growth.

Figure S1. The template used to pick sori when performing transfers and analyzing fruiting bodies.