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Fitness Consequences of Genetic and Phenotypic Chimeras in the Social Amoeba,
Dictyostelium discoideum

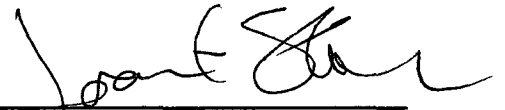
by

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ABSTRACT

Fitness Consequences of Genetic and Phenotypic Chimeras in the Social Amoeba, Dictyostelium discoideum

by

David I. Castillo

Multicellular organisms regularly eliminate unfit or harmful cells in the process of normal development. This high level of cooperation is maintained because all cells within the organism are genetically identical and thus, have the same evolutionary interests. However, there are some multicellular organisms that develop not from a single cell but from many individuals. In the case of the social amoebae, Dictyostelium discoideum, the usually solitary amoebae aggregate with nearby cells when starving to form a motile, multicellular slug that migrates to the soil surface and forms a fruiting body consisting of a ball of spores held aloft by a stalk of dead cells. These aggregations may be a mix of genetically identical individuals or of multiple clones, called genetic chimeras. Within chimeras, a conflict may arise over which cells contribute to the reproductive spores versus the dead stalk.

Previous work on D. discoideum has shown that uniclonal slugs migrate further than chimeric slugs of the same size across agar. Here we test whether this results in a fitness cost under more natural conditions. We examined migration of slugs across decaying leaves and soil as well as migration up

through layers of these substrates, closely reflecting the natural migration of D. discoideum slugs in the wild. In most trials, chimeras performed worse than single clones. Thus, chimerism in D. discoideum should produce a fitness cost likely to be important in nature.

Since D. discoideum readily mixes with multiple clones, the potential for conflict is high. Diverging evolutionary interests in chimeras could influence adaptive strategies for filling the spore and stalk roles. Here we explore the strategy for filling these roles in phenotypic chimeras, mixes of cells differing only in physical condition. We explore the fitness of D. discoideum spores and found that cells with better prospects tend to represent the subsequent generations. However, we also found that D. discoideum amoebae did not respond to the condition of cells with which they aggregate. Our results indicate that, within a genetically identical population, the differentiation of spore and stalk roles is a competition based on the condition of the individual cells.

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Chapter 1. A cost to chimerism on natural substrates

1.1 Abstract

Most multicellular organisms go through a single cell bottleneck in development, a process that ensures subsequent clonality of the cells within the individual. Selection for clonality among cells could reduce costly intra-organismal conflicts that would occur in mixtures of unrelated cells (chimeras). In Dictyostelium discoideum, the usually solitary amoebae aggregate with nearby cells when starving to form a motile, multicellular slug that may be clonal or chimeric. This slug migrates to the soil surface and forms a ball of spores held aloft by a stalk of dead cells. Previous work on D. discoideum has shown that uniclonal slugs migrate further than chimeric slugs of the same size across agar, indicating a functional cost to chimerism. Here we test whether this cost to chimerism results in a fitness cost under more natural conditions. First, we examine migration of slugs across decaying leaves or soil. Second, we examine migration up through layers of these substrates, which most closely reflects the natural migration of D. discoideum slugs to the soil surface. In most trials, chimeras performed worse than single clones. Our results indicate that chimerism in D. discoideum has a real fitness cost in the wild, likely to be compensated only by the larger size chimeras can attain in nature.

1.2 Introduction

1.2.1 Cooperation and conflict in biological systems

Multi-cellular organisms typically develop from a single cell, so that within an organism all cells are genetically identical (Bonner 1974; Dawkins 1982; Maynard Smith 1988; Maynard Smith and Szathmary 1995; Grosberg and Strathmann 1998; Michod 1999). In species where genetically distinct cells do mix, chimeras typically form only among closely related cells (Buss 1982; Grosberg and Strathmann 1998). The avoidance of low relatedness among cells predicts a reduction in costly intra-organismal conflicts. Reproductive conflict may result from competition among cells for access to the reproductive tissues (Dawkins 1982; Maynard Smith 1988; Maynard Smith and Szathmary 1995; Grosberg and Strathmann 1998; Michod 1999; Pal and Papp 2000). In addition, developmental conflict may occur if unrelated cells are unable to synchronize and communicate effectively (Wolpert & Szathmary 2002). Testing whether low relatedness causes costly within-organism conflict, however, requires one of the rare organisms in which unrelated cell lines fuse into single bodies (Strassmann et al. 2000; Maynard Smith 1988, Wolpert & Szathmary 2002). Here we use Dictyostelium discoideum, a social amoeba that readily forms chimeras with unrelated cells (Strassmann et al. 2000), to explore the costs of chimerism under natural conditions.

1.2.2 Cooperation and conflict in D. discoideum

D. discoideum is a predatory soil amoeba that is unicellular when its bacterial prey is abundant, but which aggregates when starved, to form a multicellular slug (Raper 1984, Kessin 2001). This mobile slug moves towards heat and light over and through the forest floor (Raper 1984, Kessin 2001). Upon reaching its destination, the slug differentiates into a fruiting body consisting of a stalk of about 20% dead cells that holds aloft the other 80% in a sorus of reproductive spores. The stalk raises the spores from the soil surface promoting their dispersal by passing organisms (Huss 1989, Bonner 1982). Genetic analysis of soil samples has shown that distinct clones frequently occur in very small volumes (0.2g) of soil, indicating that clones regularly mix in the wild to form chimeras (Fortunato et al. 2003). Furthermore, there is evidence of reproductive competition among clones. In chimeras of two equally proportioned clones, one clone is often over represented in the spores as compared to the stalk (Strassmann et al. 2000).

1.2.3 Benefits of mixing with unrelated individuals

Uniclinal slugs of D. discoideum migrate further than chimeric slugs of the same size (Foster et al. 2002). This supports the hypothesis that there is a functional cost to chimerism. Why then, would D. discoideum form chimeras? In D. discoideum, there is also a special benefit to chimerism because mixing with unrelated cells forms larger slugs, and larger slugs will

move further (Foster et al. 2002). This size benefit outweighs the cost of chimerism observed in same sized slugs (Foster et al. 2002).

1.2.4 Environment affects phenotype expression

However, the demonstration of the costs and benefits of chimerism was for horizontal migration on agar plates, and this raises two questions. First, although the ability to migrate horizontally is presumably useful, it still warrants direct demonstration. Vertical migration up to the soil surface has a more obvious function because this puts spores in the position for dispersal by surface organisms. Second, agar substrates can affect the observed phenotype of D. discoideum. A good example of this comes from studies of the *csA* gene, which codes for a cell adhesion glycoprotein in D. discoideum (Queller et al. 2003). This protein accumulates during aggregation and is specific for EDTA-resistant cell-to-cell adhesion. However, experiments with a *csA*-null knockout mutant on agar suggest that it is not required for successful aggregation (Harloff et al. 1989). In contrast, when tested on soil, the reduced adhesion of the *csA*-null cells proved unable to aggregate normally (Ponte et al. 1998). Such consideration raises the question of whether the cost of chimerism found in D. discoideum (Foster et al. 2002) is a true fitness detriment in the wild, motivating the experiments reported here.

We examined migration of uniclonal and chimeric slugs of D. discoideum under natural conditions. In our first set of experiments, we examined migration of slugs across substrates of decaying leaves or soil. In

our second set of experiments, we examined slug mobility up through layers of these substrates. This latter experiment closely reflects the natural migration of slugs up to the soil surface (Raper 1984, Kessin 2001).

1.3 Materials and methods

We used genetically distinct wild clones of D. discoideum, as determined by genotyping at five microsatellite loci, which were isolated from soil collected at Mountain Lake Biological Station, Virginia (Fortunato et al. 2002, 2003, Table 1). In each experiment, we obtained amoebae by plating out spores of single clones with the bacterium Klebsiella aerogenes as prey, on SM/5 agar (2g glucose, 2g peptone, 2g yeast, 1g K₂HPO₄, 1.9g KH₂PO₄, 0.2g MgSO₄, 20g agar per 1L ddH₂O). After 36 hours, before starvation and aggregation, we harvested the amoebae by washing the plates with KK₂ buffer solution (1.0g K₂HPO₄, 1.9g KH₂PO₄ in 1L ddH₂O: pH 6.1) and counted cell numbers.

The basis of all experiments was to compare slugs formed from cells of a single clone to slugs formed from a mixture of two clones (chimera). Each clonal and chimeric trial developed in a separate plate or beaker, to which we added the same total number of cells. All experimental plates and beakers used nutrient-free agar as a base for the substrates, insuring that bacterial prey do not multiply, inducing immediate amoebae starvation. In some experiments, we autoclaved soil and leaf litter and added it onto the agar. These came from a pine oak forest in the arboretum in Houston, Texas, where we have previously isolated D. discoideum.

1.3.1 Horizontal slug mobility

1.3.1.1 Migration across 10mm of substrate

We placed starved amoebae at one end of 10 cm Petri plates. The plates were stacked over each other, separated by dark cards. We then wrapped the tower of plates with black paper. At the level of each plate, at the opposite side from the amoebae, a 1 mm pinhole created a unidirectional light source towards which the slugs would migrate. We used a haphazard order in stacking the plates. In this way, we compared the distance migrated towards light by uniclonal and two-clone chimeric slugs across two natural substrates: soil and decayed leaves. We used eight clones previously collected from the field (V327A1, V319B3, V336B1, V77B, V301B1, V330D2, V324B1, V326D1, Fortunato et al. 2003, Table 1), which we compared to every possible pairwise combination (28 two-clone chimeras in total).

We placed a total of 3.6×10^6 cells (3.6×10^6 cells of one clone or 1.8×10^6 cells of each of two clones) at one end of three types of Petri dishes: agar (control), soil, and leaves. The plates with natural substrates had a strip of soil or leaves added to the surface of the agar that formed a barrier about 10mm long and 3mm high, standardized by weight (soil ~ 1g, leaf fragments ~ 0.3g) between the cells and the light source. One plate of each type was prepared for each of the 8 single clones and 28 chimeric pairs and tested on soil, leaf substrates and clean agar as a control (total plates = 108).

After 8 days, we removed the plates and evaluated slug motility by dividing them into 10 equal sections perpendicular to the light source (Foster et al. 2002). On each plate, we then counted the developed fruiting bodies in each section and calculated mean distance traveled by the slugs that formed these fruiting bodies.

1.3.1.2 Migration across 135mm of substrate

The second trial repeated the first using six clones (V56A2, V77B, V301B1, V305B3, V326D1, V330D2) and six chimeras (V56A2 x V77B, V77B x V301B1, V301B3 x V326D1, V56A2 x V326D1, V301B1 x V305B3, V305B3 x V330D2, total plates = 36). To investigate a longer distance migration, we used larger plates and more substrate (soil ~ 5g, leaves ~ 1.5g) that we spread out over the majority of the plate (135mm long), leaving 10mm of clear space at the end of the race path. We also used fewer cells per clone; pure lines had 1.4×10^6 cells and chimeras had two clones of 7.0×10^5 cells each.

1.3.1.3 Horizontal slug mobility statistics

We used two-way ANOVAs to compare the mean distances traveled by chimeras versus pure clones as well as the effects of substrate type on the results. We used one-tailed tests when testing for the cost of chimerism because we have a clear theoretical one-way prediction, chimeric slugs

migrate less far, as well as because previous studies support this prediction (Foster et al. 2002).

1.3.2 Vertical slug mobility

1.3.2.1 Migration up through 1cm and 3cm of substrate

The second set of experiments evaluated vertical migration of slugs to the surface of substrates. Since we could only observe the results of vertical migration for those slugs that reached the surface, our measure was of a threshold and not an average distance. No difference between treatments is expected if this threshold is too easy to reach (all slugs reach the surface), or too difficult (no slugs reach the surface). Thus, we set up three trials and varied conditions in later trials in response to the results of early trials. In the first trial (1.4.1), we compared slug migration upwards through soil or leaf litter of eight single clones (V55C2, V56A2, V72A2, V77B, V78C, V301B1, V326D1, V330D2) to nine chimeric pairs (V55C2 x V56A2, V56A2 x V72A2, V72A2 x V77B, V77B x V78C, V78C x V301B1, V301B1 x V301B2, V301B2 x V326D1, V326D1 x V330D2, total = 85 beakers). We added either 2.0×10^7 cells of one clone or a mixture of two clones (1.0×10^7 cells per clone) on top of non-nutrient agar at the bottom of glass beakers. We tested two depths of soil and leaves (1cm and 3cm). We sprayed 1 ml of ddH₂O into each beaker, and sealed each with Parafilm to maintain humidity. We wrapped each beaker

with black paper allowing only overhead light to enter. After leaving the beakers for 10 days, we counted the total number of fruiting bodies on the surface of the soil or leaves. For all clones and chimeras, we had strong growth on agar controls, showing that all strain could develop.

1.3.2.2 Migration up through 1cm and 2cm of substrate

Trial 1.3.2.1 revealed that few slugs were able to make it up through 3 cm of substrate. Hence, in trial 1.3.2.2, we then tested depths of 1 cm and 2 cm. In addition, we increased ddH₂O to 8ml since the substrates dried out in the first trial. We used the same clones as before except that V64D1 and V301B2 replaced V56A2 and V77B thus creating eight new chimeric mixtures (V55C2 x V64D1, V64D1 x V72A2, V72A2 x V78C, V78C x V301B1, V301B1 x V301B2, V301B2 x V326D1, V326D1 x V330D2, V330D2 x V55C2, total = 57 beakers, Table 1). Seven beakers in this trial were overgrown with fungus, and we left those samples out of the analysis.

1.3.2.3 Migration up through 1.75cm of substrate

Trial 1.3.2.3 tested only one depth of substrate (1.75 cm) based on the results of the two previous runs and used 4ml of ddH₂O. Eight clones were used: V56A1, V77B, V301B1, V301B2, V324B1, V327A1, V336B1, and V342B2 with eight chimeric mixtures (V56A1 x V77B, V77B x V301B1,

V301B1 x V301B2, V301B2 x V324B1, V324B1 x V327A1, V327A1 x V336B1, V336B1 x V324B2, V324B2 x V56A1, total = 48 beakers).

1.3.2.4 Vertical slug mobility statistics

The data were analyzed using a paired t-test that compared the number of fruiting bodies at the surface for each chimera (A/B chimera) to the average number of the two single clones when alone i.e. number of AB chimera versus $(\text{number of A} + \text{number of B})/2$. We used this test because it eliminates the effects of high variance in the number of fruiting bodies at the surface among the eight different clones.

1.4 Results

1.4.1 Horizontal slug mobility

We found a cost to chimerism in both horizontal mobility trials across all substrates (Trial 1.3.1.1, 108 plates, two-way ANOVA: $p < 0.0001$, Trial 1.3.1.2, 36 plates, two way ANOVA: $p < 0.001$; Figure 1). The substrate type did not have a significant effect on migration (Trial 1.3.1.1, $n = 108$ plates, two-way ANOVA, $p = .1113$, Trial 1.3.1.2, $n = 36$ plates, two-way ANOVA: $p = 0.2033$).

1.4.2 Vertical slug mobility

Our vertical experiment tested various successive depths. Overall, fewer chimeric slugs reached the substrate surface than their respective uniclonal slugs. Eight of our nine treatments showed that pure clones had a propensity for better vertical migration than chimeras. Out of these eight, four were significant. We observed that at intermediate depths our results were significant for both substrates. In our experiment, the depth of 1.75 cm proved to be quite advantageous for pure clones.

In Trial 1.3.2.1, clones did significantly better than chimeras in 3 cm of leaves ($n = 17$ beakers, $df = 8$, paired 1-tailed t-test: $p = 0.013$; Figure 2). The other treatments of the first trial also suggested a cost to chimerism, but were

not significant ($n = 17$ beakers each, $df = 8$, paired 1-tailed t-tests: soil at 1 cm, $p = 0.082$; soil at 3 cm, $p = 0.097$; leaves at 1 cm, $p = 0.231$).

The cost in Trial 1.3.2.2 was significant in the 1 cm soil treatment ($n = 16$ beakers, $df = 7$, for 1 cm soil, paired 1-tailed t-test: p -value = 0.021, Figure 2). Other treatments were not significant ($n = 16$ beakers, $df = 7$, paired 1-tailed t-tests: leaves at 1 cm, $p = 0.607$; soil at 2 cm, $p = 0.064$, Figure 2). One substrate had partial fungal contamination, so we used only clean samples in an unpaired test that was not significant ($n = 9$ beakers, $df = 1$, unpaired t-test, leaves at 2 cm, $p = 0.280$).

In the final trial (1.3.2.3), both treatments demonstrated decreased chimera mobility in comparison to the uniclonal samples (for soil at 1.75cm, $n = 16$ beakers, $df = 7$, paired 1-tailed t-test, $p = 0.025$; leaves at 1.75cm, $n = 9$ beakers, $df = 7$, $p = 0.024$, Figure 2).

1.5 Discussion

Chimeras were significantly less mobile than uniclonal slugs when traveling horizontally across natural substrates (Table 2, Figure 1). Established for agar substrates (Foster et al. 2002), it is equally true for natural substrates. Our first trial did not show any effect of substrate. However, it employed only a small strip of natural substrate, so we reasoned that a larger surface of soil or leaf litter might be necessary to show any possible effects on migration. Our second trial addressed this by adding a longer and wider substrate surface. When we added a more extensive substrate layer, slugs on the natural substrates moved less far than those on agar, but the result was not statistically significant. Slug mobility appears more robust than aggregation, as results from the csA experiment showed that aggregation is easier for D. discoideum on smooth agar than on soil (Ponte et al. 1998). Perhaps pointing to a benefit of aggregation; multicellular slugs are less susceptible to ill effects caused by difficult substrates than are individual cells.

The more important point is that the effect of chimerism on slowing migration was still present, showing that even on natural substrates, there is a significant migration disadvantage of mixing with unrelated clones. The remaining question is whether horizontal migration matters for fitness. It seems highly likely that it does. Slugs use resources to migrate and migration is a highly ordered response to specific cues such as light and ammonia, both

suggesting that it is often adaptive. Slugs that are able to migrate farther would have a greater ability to find optimal fruiting sites. However, to address this concern, we also tested vertical migration through soil or leaves, where failure to migrate far enough carries the more obvious cost of either no spore production or perhaps production of spores that remain buried in the substrate.

Chimeras were either the same or worse than single clones in their ability to migrate vertically to reach the soil surface (Table 2, Figure 2). The lack of difference between chimeras and clones in some trials is presumably due to the deepest substrates preventing most slugs from making the surface, while the shallow ones allow all to reach the surface. If conditions are sufficiently easy or difficult, either all or none of the slugs will reach the surface, irrespective of chimerism. However, the cost manifests under intermediate conditions, and chimeras never performed better than single clones. In nature, migrating D. discoideum slugs face a highly variable range of soil depths and moistures (Raper 1984, Kessin 2001) so that the cost is likely to affect them some of the time. On average, therefore, chimerism is likely to carry a real fitness cost in nature.

In D. discoideum, this cost seems outweighed by the benefits of mixing with non-kin because this allows amoebae to form larger aggregations (Foster et al. 2002). A larger aggregation can form a larger slug that can migrate farther than a smaller one (Foster et al. 2002). This size effect means that cell density may be another important variable influencing the cost of chimerism.

In our vertical experiments, 10^7 cells had a critical depth of 1.75cm. At this depth, we were able to tease apart the difference between the pure clones and their chimeras. At depths more shallow than that, we could see that chimerism was not relevant to our measured fruiting success. For other cell densities, the critical depth will no doubt vary. At a much higher cell density, the larger slugs might all succeed at 1.75 cm, but the disadvantage of chimeric slugs may still apply at 3 cm. The opposite extreme should also hold. At much lower densities, the smaller slugs may all fail at 1.75 cm, and the disadvantage of chimerism would only apply at shallower depths.

In summary, the occurrence of a fitness reduction in chimeras of D. discoideum in our realistic environment supports the idea that there is an intrinsic cost when genetically distinct cells mix. We have shown that the effect of environment on selection on chimerism will be substantial in D. discoideum. The intrinsic cost should conditionally translate into a real fitness cost in nature, depending on the circumstances such as depth and cell density. However, the circumstances where the cost is manifest are also circumstances where large slug size is important, so that joining in chimeras may still be better than going it alone.

1.6 References

- Bonner, J. T. 1974. *On development*. Harvard, Mass: Harvard University Press.
- Bonner, J. T. 1982. **Evolutionary strategies and developmental constraints in the cellular slime moulds**. *Am. Nat.* **199**, 530-552.
- Buss, L. W. 1982. **Somatic cell parasitism and the evolution of somatic tissue compatibility**. *Proc. Natl. Acad. Sci. USA* **79**, 5337-5341.
- Dawkins, R. 1982. *The extended phenotype. The long reach of the gene*. Oxford: Oxford University Press.
- Fortunato, A., Queller D.C., Strassmann J.E. 2003. *Mol. Ecol.* **12** (4): 1031-1038.
- Foster, K. R., Fortunato, A, Strassmann, J.E. & Queller D.C. 2002. **The costs and benefits of being a chimera**. *Proceedings of the Royal Society of London, Series B.* **269**: 2357-2362.
- Grosberg, R. K. & Strathmann, R. R. 1998. **One cell, two cell, red cell, blue cell: the persistence of a unicellular stage in multicellular life histories**. *Trends Ecol. Evol.* **13**, 112-116.
- Harloff, c., Gerish, G. & Noegel, A. 1989. *Genes Dev.* **3**, 2011-2019.
- Kessin, R. H. 2001. *Dictyostelium. Evolution, Cell Biology, and the Development of Multicellularity*. Cambridge: Cambridge University Press.

- Maynard Smith, J. 1988. **Evolutionary progress and levels of selection.** In *Evolutionary Progress* (ed. M. H. Nitecki), 219-230. Chicago: University of Chicago press.
- Maynard Smith, J. & Szathmary, E. 1995. *The Major Transitions in Evolution.* New York: Freeman.
- Michod, R. E. 1999. *Darwinian Dynamics. Evolutionary Transitions in Fitness and Individuality.* Princeton, NJ: Princeton University Press.
- Pal, C. & Papp, B. 2000. **Selfish cells threaten multicellular life.** *Trends Ecol. Evol.* **15**, 351-352.
- Ponte, E., Bracco E., Faix, J., Bozzaro S. 1998. **Detection of Subtle Phenotypes: The Case of the Cell Adhesion Molecule csA in *Dictyostelium*.** *Proc. Natl. Acad. Sci. U.S.A.* Vol. 95, No. 16, 9360-9365.
- Queller, D.C., Ponte, E., Bozzaro, S. and Strassmann, J.E. 2003. **Single-gene greenbeard effects in the social amoeba, *Dictyostelium discoideum*.** *Science* **299**:105-106.
- Raper, K. B. 1984. *The Dictyostelids.* Princeton, NJ: Princeton University Press.
- Strassmann, J. E., Zhu, Y. & Queller, D. C. 2000. **Altruism and social cheating in the social amoeba *Dictyostelium discoideum*.** *Nature* **408**, 965-967.
- Wolpert L., Szathmary E. 2002. **Multicellularity: Evolution and the egg.** *Nature* **420**, 745-745.

1.7 Tables

Table 1 Clones used in the experiments.

Trial	1.3.1.1	1.3.1.2	1.3.2.1	1.3.2.2	1.3.2.3
V55C2			X	X	
V56A1					X
V56A2		X	X		
V64D1				X	
V72A2			X	X	
V77B	X	X	X		X
V78C			X	X	
V301B1	X	X	X	X	X
V301B2			X	X	X
V305B3		X			
V319B3	X				
V324B1	X				X
V326D1	X	X	X	X	
V327A1	X				X
V330D2	X	X	X	X	
V336B1	X				X
V342B2					X

Table 2 Summary of main experimental conditions and results. Yes means the result was statistically significant (see text and figures).

Trial	Migration	Substrate details	Water added?	Soil result significant?	Leaf result significant?
1.3.1.1	Horizontal	10 mm length	n/a	yes	yes
1.3.1.2	Horizontal	135 mm length	n/a	yes	yes
1.3.2.1	Vertical	3 cm depth	1 ml	no	yes
1.3.2.1	Vertical	1 cm depth	1 ml	no	no
1.3.2.2	Vertical	1 cm depth	8 ml	n/a	no
1.3.2.3	Vertical	1.75 cm depth	4 ml	yes	yes

1.8 Figures

Figure 1 Migration of uniclonal and chimeric slugs horizontally across natural substrates. Trial 1.3.1.1 showed that uniclonal slugs traveled consistently farther regardless of substrate (108 plates, two way ANOVA: $p < 0.0001$). Trial 1.3.1.2 (b) showed that the reduction in mobility also occurred over a larger terrain (36 plates, two way ANOVA: $p = 0.0003$). White columns are pure clones, while black columns are chimeras. * indicates $p < 0.001$ (see test for details).

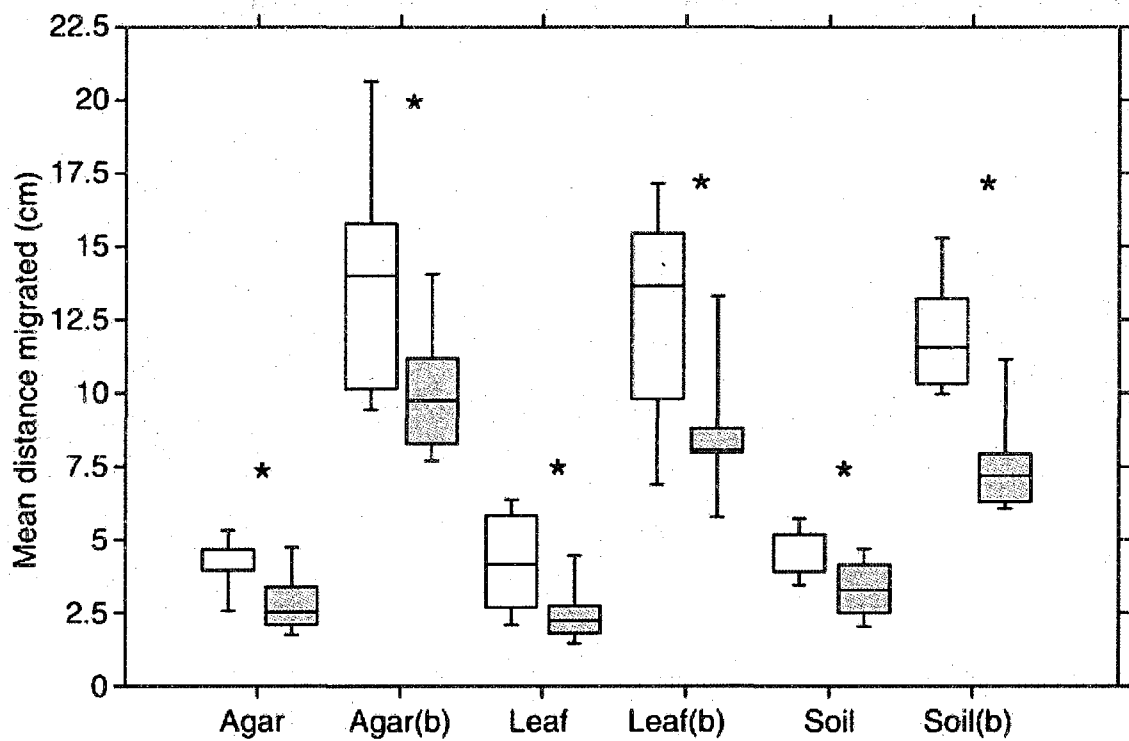
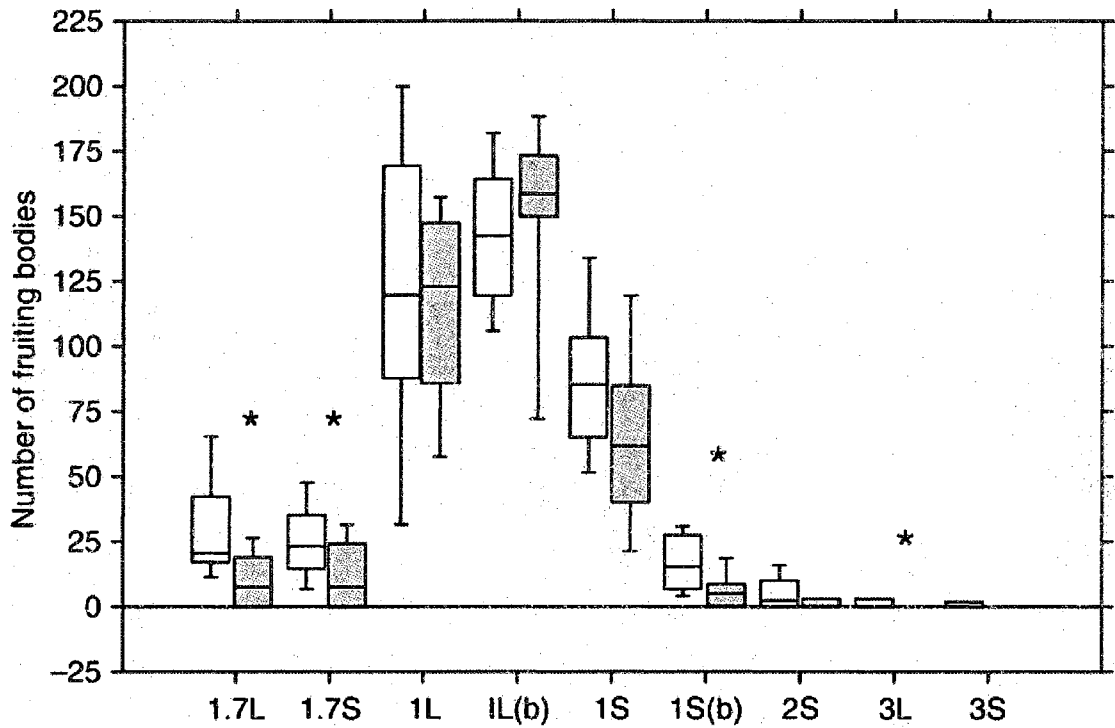


Figure 2 Number of fruiting bodies at the surface as after vertical migration of uniclonal and chimeric slugs through differing depths of natural substrates. White columns are pure clones, while grey columns are chimeras. Treatments are designated by a number representing the depth in cm followed by S for (soil) and L for (leaves). S1 and L1 were each done twice, so a (b) is used to designate between the trials. Asterisks indicate $p < 0.05$ for the hypothesis that uniclonal treatments have equal fruiting bodies as chimeras (see text for details).



Chapter 2 Fitness consequences of phenotypic chimeras

2.1 Abstract

Multicellular organisms consist of identical cells with identical evolutionary interests and maintain a high level of cooperation. However, there are some multicellular organisms that develop from aggregation where individuals are not always related. These systems allow for the exploration of evolutionary strategies within the context of both selfish interests and highly cooperative behavior. The social amoeba, Dictyostelium discoideum, is one example; the aggregated multicellular slug is derived from individual amoebae and develops into a sorocarp consisting of a dead stalk and a sorus of reproductive spores. In aggregations between unrelated cells, individuals should evolve strategies to gain the advantages of cooperation but also obviate the costs when possible.

To understand what strategies D. discoideum uses to allocate cells to the spore and stalk roles, we used a single genetic clone and created three conditionally distinct cell lines; glucose-starved cells, acid-stressed cells and control lines grown in standard medium. We explored whether cell condition, in the absence of genetic competition, affects the differentiation of cells into spores. When mixed at a 1:1 ratio with our control cells, weaker cells were under-represented as spores. To deduce what selective pressures may be influencing the strategy, we also explored if cell condition causes a measurable fitness change in the resulting spores. Treated cells produced spores that, compared to the control, germinated equally but were slow to

establish a new generation. We also compared the spore production of these three cell types when alone to when mixed with cells of a different condition to see if D. discoideum cells employ a strategy to take advantage of fitness differences of spores by increasing production when mixed with weaker cells. We found that healthy cells did not adjust their spore production when mixed with weaker cells but found that treated cells produced fewer spores when alone, accounting for the skewed representation in mixes. So, the greater contribution to fertile spore cells by control cells is a reflection of their overall health and not of a strategy that only appears when confronted with cells of a different condition.

2.2 Introduction

2.2.1 Cooperation and conflict in biological systems

Competition between genetically distinct individuals for limited resources or reproductive rights selects for selfish strategies. Just as a male lion may take away a kill from unrelated female hunters and their unrelated young, selfish behavior can be found in many biological systems. Even within colonies of eusocial organisms in which non-reproductive worker castes evolved to rear the young of the related reproductive castes, conflict still arises (Queller 2003). When a honeybee queen leaves to establish a new colony, her daughters engage in a fight to the death to claim the old colony as their own. These potential queens, only half-sisters to each other, will pass on more genes by becoming the new queen than by successfully helping a sister do so (Gilley 2001). Thus, although social groups evolve altruistic behaviors, selfish strategies will often occur when an individual advantage is to be had even if it diminishes group productivity.

Multicellular organisms reduce potential internal conflict by undergoing single celled bottlenecks every generation, ensuring all cells within the individual are genetically homogenous and thus, share evolutionary interests (Smith & Szathmary 1995, Grosberg & Strathmann 1998). The ubiquitous role of programmed cell death in the development of most animals exemplifies this shared interest. Programmed cell death requires a set of genetic instructions

that, when turned on, cause a cell to autonomously die. In spite of the individual cost, cell suicide is beneficial to the whole organism under many different circumstances (Raff 1992). Cells of various types may be produced in excess or may have previously served a function that is no longer needed (Lockshin 1981). The development of physical structures is sometimes accomplished by the pruning of tissues such as between developing digits in amniotes (Hammar 1971). In the case of the adaptive immune system, lymphocytes that fail to produce antigen-specific receptors are of no use while those that produce self-reactive receptors are directly harmful to the whole organism; both classes are routinely eliminated (Cohen 1991, Golstein 1991). Multicellular organisms can maintain a high level of cellular cooperation because almost all cells, whether somatic or germ-line, are genetically identical. However, there are some multicellular organisms that develop not from a single cell, but from aggregation of many individuals. These natural systems allow for direct examination of the tension between cooperation for the good of the whole organism and competition of individual interests by the cells that constitute the organism.

2.2.2 Cooperation and conflict in D. discoideum

The social amoeba Dictyostelium discoideum is one such system, primarily existing as a solitary amoeba living on the forest floor and feeding on soil bacteria (Bonner 1967). When starved, however, D. discoideum amoebae send out a cyclic AMP signal that attracts nearby amoebae into aggregations

that, in turn, develop into multicellular slugs capable of migration (Raper 1940, Kessin 2001). A slug is made up of cells that fall into two major categories, pre-stalk and pre-spore. Once a slug migrates to a suitable location, the approximately 20-30% of cells specified as pre-stalk cells die to form a stalk that holds aloft the remaining pre-spore cells, which produce a sorus consisting of viable spores (Bonner 1967, Raper 1984). The stalk aids in spore dispersal and lifts the sorus up above the hazards of the soil to allow time for later germination (Bonner 1982, Kessin 2001). All starving amoebae have the potential to become spores and all cells within a slug can fill either role (Raper 1940, Shaulsky 1993).

Distinct clones of *D. discoideum* co-occur on a small scale in nature and form mixtures, called chimeras, in lab experiments (Strassmann *et al.* 2000, Fortunato *et al.* 2003). Since genetically distinct clones can form a single multicellular organism, there is a potential for internal conflict (Strassmann *et al.* 2000). Indeed, when different clones co-aggregate, some clones tend to become over-represented in the pre-spore region of the slug as well as in the final sorus (Strassmann *et al.* 2000, Fortunato *et al.* 2003, Ennis *et al.* 2000). Furthermore, mixtures of different clones produce slugs that are reduced in their ability to migrate as compared to clonal slugs, further suggesting a conflict between the unrelated individuals (Foster *et al.* 2002, Castillo *et al.* 2005). Still, the fact that distinct clones mix implies that perhaps the benefits of mixing outweigh the costs. A possible benefit to mixing is that

large slugs made up of multiple clones will migrate farther than smaller slugs made up of single clones (Foster *et al.* 2002).

It is also possible that conflict between clones does not affect the stability of this cooperation because cheating is effectively suppressed. One way this could occur is if an environment of high relatedness aids in selection against cheaters (MaynardSmith 1989, Grosberg & Strathmann 1998, Queller 2000). Initial indications are that relatedness in nature is high; measurements by two different methods showed 77% and 92% of wild fruiting bodies collected from dung were clonal, indicating that relatedness was high enough to limit the spread of many genetic cheaters (Gilbert *et al.* 2007). Another way individual exploitation would be limited is by the linkage of likely genetic paths of cheating to essential functions. In fact, studies on a genetic cheater, a *dimA* knockout, has shown that although knockouts are able to ignore signals for differentiating into pre-stalk cells, when mixed with wild-type cells the ability of these mutant cells to produce spores is reduced, thereby undermining any benefit to ignoring pre-stalk signals (Foster *et al.* 2004). Furthermore, if cells can recognize clonemates, then they might use this ability to favor clonemates over non-clonemates within a chimera, or to sort away from non-clonemates. Recognition was observed in a related species D. purpureum, where although distinct clones aggregate together, unrelated cells tend to sort into different fruiting bodies (Mehdiabadi *et al.* 2006). Although no study has been published on sorting by D. discoideum, there are other ways in which cells may adjust their behavior in order to gain a benefit.

For example, cells may produce a greater number of spores or fewer stalk cells when in contact with cells of a different type.

2.2.3 Social context can influence selective pressures

The social context of D. discoideum in nature is complicated.

Genetically distinct clones readily co-aggregate even though they experience a cost to migration, and they co-occur in small areas of soil in nature but maintain a high relatedness when observed as fruiting bodies on dung.

Genetic cheaters may experience pleiotropic costs further allowing for stable cooperation to evolve. As much as is understood about their natural social context, it is still unclear how important natural chimerism is in the evolution of D. discoideum.

The social context of D. discoideum can influence selective pressures in several important ways. First, within individual fruiting bodies containing multiple clones, selection could favor clones that contribute more than their share to the sorus or reduce their contribution to the stalk. A social context typically consisting of multiple clones would not necessarily select for the best spores but simply for those that are best at entering the sorus. In a clonal environment, the evolution of sterile stalk cells would be favored by kin selection since the evolutionary interests of the cells relegated to the stalk match that of the cells entering the spores (Hamilton 1964 a,b). Cells may gain from cooperating by increased dispersal via slug migration and stalk

formation, but they should still evolve strategies that allow them to gain the benefits of cooperation while obviating the costs as much as possible.

Although cooperating cells might be genetically identical, they may not necessarily be identical in many stochastic respects. Cells can differ in size, energy stores, expression and recognition of signals, nutritional history, and cell cycle position. So, in fruiting bodies of pure clones, selection should be also expected to act on these differences, ensuring the fittest spores for the next generation. There is evidence that D. discoideum apportions cell types based on the relative condition of cells. DIF-1, an intercellular signal molecule, directs cells to differentiate into a major subtype of pre-stalk cells (Shaulsky 1996, Thompson and Kay 2000 a,b). Cell sensitivity to DIF-1 is based on previous growth history and cell cycle position (Takeuchi 1969, Leach *et al.* 1973, Maeda and Maeda 1974). Specifically, genetically identical cells of D. discoideum show a bias of spore differentiation based on the nutritional history of the amoebae; in mixes of cells grown with and without glucose, cells grown with glucose tend to be preferentially allocated to the pre-spore sections of the slug and in the fruiting body (Leach *et al.* 1973, Tasaka *et al.* 1981, Blaschke *et al.* 1986). Presumably, these results show that the multicellular slug allocates the best cells into the reproductive sorus (Khare & Shaulsky 2006). However, it is unknown if these 'healthy' cells actually produce spores of a better quality than 'unhealthy' cells. It is also unknown if a general indicator of amoeba condition triggers the sensitivity to pre-stalk signals or if the result is specific to glucose-starved cells.

Understanding the behavior of D. discoideum cells differing only in physical condition may give us an insight to how D. discoideum has adapted to cooperation in nature. Here we use a single clone to understand what strategies D. discoideum cells utilize to fill the spore and stalk roles. If cells are employing a strategy in which allocating cells with the best prospects to the sorus is the most important aspect, we expect 'healthy' cells to produce spores of a higher quality. This should most likely occur if the strategy evolved in a typically clonal context. If *D. discoideum* is regularly in a chimeric context, a fixed strategy to ignore pre-stalk signals may be more important than a cell's future prospects. In that case, we expect 'healthy' cells to not necessarily produce spores of a higher quality, but to be better at entering the sorus. Furthermore, D. discoideum cells may have evolved mechanisms for adjusting their behavior depending on the condition of co-aggregating cells.

We hypothesize that cells with the best prospects should be preferentially allocated to the sori. We also hypothesize that if D. discoideum cells evolved a strategy to adjust their behavior with regards to their social context, we will observe an exaggerated difference between the spore production of 'healthy' and 'unhealthy' cells when developed in mixes as compared when each is grown and developed alone.

2.2.4 Phenotypic chimeras

We treated a single clone of D. discoideum cells with media modified in two ways; we used cells that had been grown without glucose and cells grown

in an acidic medium to produce 'unhealthy' cells while using cells in standard liquid media (Glucose-fed, pH ~6.7) as 'healthy' cells. We used labeling techniques to measure the relative contribution to the fruiting bodies by cells of differing conditions in equal mixes, called phenotypic chimeras. We tested whether cells that are more likely to contribute to the stalk produce less fit spores by measuring the germination of resulting spores and the proliferation of subsequent generations. Finally, we compared the spore production by these cell types in phenotypic chimeras to their spore production when developed alone.

2.3 Materials and methods

2.3.1 Strains

For all assays we used an axenic lab strain, AX4, which was sequenced by the Dictyostelium discoideum genome project. For mix assays with cells grown in HL-5 (G-) we also used Ax4 transformed with GFP (green fluorescent protein) labeling.

2.3.2 Cell preparation

We began AX4 growth in 20ml of HL-5 media (bactopeptone #2 10g, yeast extract 5g, glucose 10g, Na₂HPO₄ .35 g, KH₂PO₄ 0.35, 100x trace elements 1ml, to pH 6.4-6.6) in a tissue culture plate. When the spores hatched and divided to cover the bottom of the plate, we used a pipette to harvest the cells and transferred them to glass flasks with 50ml of HL-5. A single flask of cells was used to begin each replicate of all assays. We transferred cells from each flask once a day to a new flask and brought back to the starting density of 1×10^6 cells/ml. After at least two transfers, we used each flask of AX4 cells to seed additional populations in modified media that were transferred and observed as described above.

2.3.3 Modification of media

For all assays, we modified the standard HL-5 media to produce two modified growth conditions, HL-5 (G-) and HL-5 (pH 4.5). HL-5 (G-) was prepared by eliminating glucose from the standard recipe, while the HL-5 (pH 4.5) modified the standard recipe only by the further addition of 400ul H₂PO₄ to each liter of solution to pH ~4.5. Our experiments compared cells of the parent strain, AX4 as our 'healthy' control and AX4 cells that have been grown in modified solutions AX4 (G-), AX4 (pH4.5) as our 'unhealthy' treatments.

2.4 Data collection

2.4.1 Cell doubling rate

For all of our populations, we used doubling time as a proxy for condition. We estimated population growth by the equation: $T_d = (t_2 - t_1) * \log_{10}(2) / \log_{10}(q_2/q_1)$, where the doubling time (T_d) is defined as the period of time ($t_2 - t_1$) required for the population (q_1 is the initial density of 1×10^6 , q_2 is the density at the time of counting) to double in size. We tracked the growth of AX4 cells grown in standard liquid medium (HL-5, pH 6.4-6.7), and when we used these cells to seed populations in two modified HL-5 media, we confirmed that the modified solutions produced conditionally distinct amoebae by tracking doubling times for each group every day.

2.4.2 Spore fitness assays

We measured spore production, spore germination, and the growth rate of subsequent generations of AX4 and the two conditionally different populations, AX4 (G-) and AX4 (pH 4.5). We began these assessments by isolating $\sim 8 \times 10^7$ cells from flasks of AX4, AX4 (G-) and AX4 (pH 4.5). We suspended the cells in 50ml standard KK2 buffer (3.8mM K_2HPO_4 , 16.5mM KH_2PO_4), centrifuged three times at 1000 rpm (?rcf) for three minutes to

remove all remaining nutrient solution and resuspended cells to a concentration of 4×10^7 cells per milliliter in KK2 buffer. For each of the three types of cells, we put ten aliquots of 2×10^6 cells on two nitrocellulose filters (5 per filter) and allowed the cells to develop. We were not able to control for the fact that some cells seemed to be able to divide after we placed them down on the filters, and that this might affect our results. The resulting fruiting bodies were collected in 5 ml of KK2 buffer. Using a hemocytometer to count spore density, we calculated the number of resulting spores. Spore production of each group was determined by dividing the number of spores collected by the number of cells that created the fruiting bodies. We repeated this procedure five times. For our assays with AX4 (pH 4.5) we suspected that one point was an experimental error since it was a maximum outlier by 1.7 standard deviations; so we replaced the point by the next highest value of that set. This process of winsorizing the data produced the significant effect reported above (Sokal and Rohlf 1995). Without the adjustment of the outlier, our results were in the same direction but not statistically significant (student's t test: $n = 5$, $t = 0.90$, $p = 0.198$).

To assess germination efficiency we plated out approximately 100 spores per plate on six 100mm Petri plates containing SM/5 agar (2g Glucose, 2g Bactopeptone, 2g yeast extract, 0.2g $MgSO_4$, 1.9g KH_2PO_4 , 1g K_2HPO_4 , 20g agar in 1L H_2O) with 300 μ l of the bacteria *Klebsiella aerogenes* (KA) as food. As the spores germinated we scored the number of new

colonies, indicated by clearings in the bacterial lawn. We repeated this procedure six times.

To assess the new colony growth of each group, we marked eight segments on each of six plates prepared as described above. We then measured the diameter of an isolated clearing nearest the center of each segment, for a total of 48 clearings measured. We repeated this nine times.

2.4.3 Phenotypic chimera assays

For phenotypic chimera assays with AX4 (G-), we prepared AX4-GFP cells in the same manner as the parent strain, but maintained the GFP transgene by use of 2.5 μg G418 (an aminoglycoside antibiotic also known as Geneticin) to which the AX4-GFP strain was engineered to be resistant. The AX4-GFP was also used to seed a population in HL-5 (G-) media, AX4-GFP (G-).

We began the phenotypic chimera assay by isolating 8×10^7 cells from flasks of AX4 and AX4 (G-) as well as AX4-GFP and AX4-GFP (G-). We suspended the cells in 50ml standard KK2 buffer, centrifuged and resuspended cells to a concentration of 4×10^7 cells per milliliter in KK2 buffer as described above. We mixed equal numbers of AX4 with AX4-GFP (G-) cells and AX4-GFP with AX4 (G-) cells and utilized pure AX4-GFP and pure AX4-GFP (G-) controls to adjust counts for non-glowing GFP cells in the mixes. We used the fluorescence of GFP to follow the conditionally different

cells through development during co-aggregation. We also mixed equal proportions of AX4 with AX4-GFP and AX4 (G-) with AX4-GFP (G-) as a control for any labeling effect. We used pure AX4 and AX4 (G-) cells as well as pure AX4-GFP and AX4-GFP (G-) to observe the effect of condition on unmixed cells developed alone. For every sample we plated 2×10^6 cells (in 50 μ l), on individual nitrocellulose filters (0.8 μ m /type AABG Millipore brand) sitting on non-nutrient agar (2% agar by weight). The resulting fruiting bodies of each group were collected in 1 ml of KK2 buffer. We calculated the number of resulting spores of all groups with a hemocytometer as described above and used fluorescence microscopy (E1000 florescent microscope with MetaMorph® imaging software) to count GFP spores in mixes. We repeated this procedure two times.

For phenotypic chimera assays with AX4 (pH 4.5), we were unable to consistently grow a GFP strain in acidic media. In lieu of the GFP strain, we used CellTracker™ Green CMFDA on the day of the mix assay to dye both AX4 and AX4 (pH 4.5) cells. We followed the manufacturer's recommended protocol to label the cells, and our working solution was 25 μ M dye. Once we labeled the cells, we were able to utilize the same procedures for mixing, fluorescence and microscopy as in the GFP assay. We also repeated this assay twice.

2.5 Results

2.5.1 Cell doubling rate

We measured the growth of each population at the time of each daily transfer and these values were used to determine the health of all cell populations. On the days of the assays, our AX4 control (hereafter called “control”) took less time ($8.09 \pm \text{SE } 0.23$ hrs) to double in number than our ‘unhealthy’ cells grown without glucose, (glucose-starved), ($12.63 \pm \text{SE } 0.62$ hrs) or cells grown in an acidic solution, (acid-stressed), ($12.51 \pm \text{SE } 0.86$ hrs). When we compared our control to glucose-starved cells and to acid-stressed cells on the day of the assays (Fig 1a,b), there was a significant increase in doubling time for the treated cells (Control vs. glucose-starved, two way ANOVA: $df = 1$, $p < 0.0001$; Control vs. acid-stressed; two way ANOVA: $df = 1$, $p < 0.0001$). Since our each of our assays were replicated on different days, we tested the effect of replicates and found that they were not significantly different than each other, showing that our liquid medium treatments did not affect our cells differently with respect to the day of the assays (Control vs. glucose-starved; two way ANOVA: $df = 12$, F ratio = 0.46, $p < 0.9030$; Control vs. acid-stressed; two way ANOVA: $df = 12$, F ratio = 1.54, $p = 0.2317$).

2.5.2 Allocation of cells to spore role in phenotypic chimeras

We mixed our control cells with glucose-starved cells and with acid-stressed cells, each at a 1:1 cell ratio and allowed for aggregation and fruiting body formation and compared the relative proportions of our control and treatments found as spores to the expected proportion of 1:1. Figures 2 and 3 show that for each individual mix, glucose-starved cells and acid-stressed cells were underrepresented as spores when each was combined with our control cells (single sample chi-square test: glucose-starved mixed with the control: {control labeled mixed with G-, G- labeled mixed with control} for each of 3 replicates, $X^2 = \{40.5, 58.4\} \{29.30, 22.99\} \{103.81, 11.78\}$, $df = 1$, $p < 0.05$ for all tests; acid-stressed mixed with control: {control labeled mixed with pH 4.5, pH 4.5 labeled mixed with control} for each of 3 replicates, $X^2 = \{48.5, 29.69\} \{10.29, 11.67\} \{14.37, 13.77\}$, $df = 1$, $p < 0.05$ for all tests). Since we used cell-labeling techniques to observe the amoebae in fruiting bodies, we also tested the effect of labeling on spore allocation and found no significant effect (single sample chi-square test: {control mixed with control labeled, G- mixed with G- labeled} for each of 3 replicates, $X^2 = \{1.88, 2.60\} \{0.80, 0.01\} \{2.11, 0.93\}$, $df = 1$, $p > 0.05$ for all tests; {control mixed with control labeled, pH 4.5 mixed with pH 4.5 labeled}, $X^2 = \{2.23, 3.12\} \{1.16, 2.43\} \{1.62, 0.73\}$, $df = 1$, $p > 0.05$ for all tests). Since our labeling controls did not have an effect on our control mixes and our experimental mixes between control and treated cells were all independently significant, we

merged the labeled and unlabeled results to represent all data in terms of condition and show that, glucose-starved cells experienced a decreased spore contribution of $13.3 \pm \text{SE } 2.2 \%$ when mixed with control cells and allowed to form social fruiting bodies (single sample t test: $df = 5$, $t = -4.35$, $p < 0.01$) while acid-stressed cells showed a reduced spore allocation of $8.9 \pm \text{SE } 1.5 \%$ when mixed with control cells (single sample t test: $df = 5$, $t = -8.91$, $p < 0.0001$).

2.5.3 Spore germination

Spores from glucose-starved cells germinated at a rate of $49.12 \pm \text{SE } 5.62 \%$, this was not statistically different from the rate of germination of our controls, $51.77 \pm \text{SE } 7.56 \%$ (two way ANOVA, Tukey Honestly Significant Difference not significant at $\alpha = 0.05$). Spores from acid-stressed cells germinated at a rate of $50.37 \pm \text{SE } 5.97 \%$ and this was also not different from our controls (two way ANOVA, Tukey HSD not significant at $\alpha = 0.05$)(Fig 4). Furthermore, the day of replicate did have a significant effect on germination across all cell types (two way ANOVA: $df = 5$, $p = 0.0101$).

2.5.4 New generation cell proliferation

Spores derived from our control cells hatched and then divided while consuming bacteria. The process of consuming the bacterial lawn clears an area of agar that can be measured as a proxy for new generation cell

proliferation. Our controls had a mean clearing area of $7.43 \pm \text{SE } 0.82 \text{ mm}^2$ while spores derived from glucose-starved and acid-stressed cells established significantly smaller clearings of mean areas $6.42 \pm \text{SE } 0.76 \text{ mm}^2$ and $6.20 \pm \text{SE } 0.78 \text{ mm}^2$, respectively (two way ANOVA; control vs. glucose-starved, Tukey HSD significant at $\alpha = 0.05$; control vs. acid-stressed, Tukey HSD significant at $\alpha = 0.05$) (Fig. 5). The day of replicate had an effect on cell proliferation across cell types (two way ANOVA: $df = 8$, $p = 0.0101$).

2.5.5 Spore production

The percentage of control cells that produced spores was $88.96 \pm \text{SE } 12.4$. The percentage of glucose-starved cells that produced spores was $58.68 \pm \text{SE } 8.89$ and was significantly lower than that of the control (two way ANOVA; control vs. glucose-starved, Tukey HSD significant at $\alpha = 0.05$). The spore production of acid-stressed cells was $51.12 \pm \text{SE } 6.66 \%$ and this was also significantly lower than that of our control (two way ANOVA; control vs. acid-stressed, Tukey HSD significant at $\alpha = 0.05$) (Fig. 6). The day of replication had an effect on cell production across cell types (two way ANOVA: $df = 4$, $p = 0.0019$).

Our cell labeling allowed us to examine if the unhealthy cells showed a change in spore production depending on whether they were co-aggregating with healthy cells and vice versa. Using data from the phenotypic chimera

assays reported before, we measured spore production of our control and treated cells and found that our healthy cells did not produce a significantly different proportion of spores when alone as compared to when mixed with the unhealthy cells (two way ANOVA: $df = 5$, $p = 0.719$) (Fig. 7). Glucose-starved cells showed no difference in spore production when developed alone as compared to when mixed with our control (two way ANOVA: $df = 2$, $p = 0.1383$) (Fig. 8). Acid-stressed cells also showed no difference in spore production when alone as compared to when mixed with healthy control cells (two way ANOVA, $df = 2$, $p = 0.8656$) (Fig. 9). The effect of replicate day on spore production was not significant in these labeling assays (two way ANOVA: control alone vs. when mixed with unhealthy, $df = 5$, $p = 0.058$; glucose-starved when alone vs. when mixed with control, $df = 2$, $p = 0.07$; acid-stressed when alone vs. when mixed with control, $df = 2$, $p = 0.201$).

2.6 Discussion

Biological altruism, where one individual gives up fitness in order to increase the fitness of another, can be favored by kin selection. The social amoeba Dictyostelium discoideum provides a clear example: some cells give up their lives to create a stalk that enhances the dispersal of other cells, usually their clonemates (Strassmann *et al.* 2000)). Such altruism may be sensitive to condition: unhealthy cells with poor prospects should give way to, or lose a competition against, their healthy clonemates (Khare and Schaulsky 2006). From the standard medium (HL-5), we created two modified 'unhealthy' solutions to produce cells that divided more slowly. Indeed, we found that cells in poor condition tended to be under-represented in the spores when mixed with our control cells in phenotypic chimeras. To further ascertain what strategy D. discoideum amoebae use to fill the stalk and spore roles, we measured the future prospects of spores from cells of these different conditions. We found that unhealthy cells produced spores that germinated at the same rate as our controls and thus, were as viable as spores from healthy cells. However, we observed that spores from unhealthy cells hatched out and proliferated on bacteria into clearings that were measurably smaller than those from our healthy controls. Presumably, these new groups of cells were reduced in their ability to feed, go through early cell divisions, or had impaired cell movement due to the condition of the spores

from which they hatched. We also noted that D. discoideum cells could adjust their spore production when co-aggregating with cells of a different condition as a strategy to allocate the best spores to the sori. We noted that although both unhealthy cell types produced fewer spores than our healthy control when alone, neither unhealthy cell type adjusted their spore production when aggregating with healthy cells. Likewise, healthy cells did not produce a greater proportion of spores when mixed with unhealthy cells as when alone.

We determined that amoebae in better condition are more likely to represent the next generation. We also tested the presumption that the cells most likely to represent the spores were actually more fit. Indeed, our 'unhealthy' cells had poorer future prospects, a reduced ability to proliferate after spore germination, but were also reduced in their ability to produce spores in the first place. Surprisingly, the filling of spore and stalk roles was not the result of an active strategy to allocate the cells with the best prospects to the spore. In our experiments, the reduced solitary spore production of our unhealthy lines explained their under-representation in the spores in mixtures.

We hypothesized that if D. discoideum cells evolved in a typically clonal environment they could employ a strategy to allocate the spores with the best prospects to the sorus. However, D. discoideum was not actively filling roles based on condition rather, the condition of cells produced the effect of filling the spore role with cells of better prospects. This fits with the idea that D. discoideum is typically clonal in nature where, if condition is an accurate predictor of future success and if health affects the inherent spore

production, the strategy to produce as many spores as possible could be favored. The result is that the next generation will consist of genetically identical cells that have a better chance of survival owing to their condition. However, this could also serve well when forming genetic chimeras; producing as many spores as possible will be beneficial when mixing with another clone because it ensures that a clone's fittest cells will differentiate into spores as much as possible. Thus, the spore/stalk differentiation in chimeras may be viewed as a competition where clones that are regularly in better condition will win.

2.7 References

- Blaschke A, Weijer C, MacWilliams H: ***Dictyostelium discoideum*: Cell-type proportioning, cell-differentiation preference, cell fate, and the behavior of anterior-like cells in Hs1/Hs2 and G+/G- mixtures.** *Differentiation* 1986, **32**, 1-9.
- Bonner J T 1952 The pattern of differentiation in amoeboid slime molds; *Am. Nat.* **86** 79–89
- Bonner JT: *The cellular slime moulds*, 2nd ed. Princeton University Press (1967).
- Bonner JT: **Evolutionary strategies and developmental constraints in the cellular slime moulds.** *Am. Nat.* 1982, **119**:530-552.
- Buss LW: **Somatic cell parasitism and the evolution of somatic tissue compatibility.** *Proc. Natl. Acad. Sci.* 1989, **79**: 5337-5341.
- Castillo DI, Switz GT, Foster KR, Queller DC, Strassmann JE: **A cost to chimerism in *Dictyostelium discoideum* on natural substrates.** *Evol. Eco. Res.* 2005, **7**: 263-271.
- Cohen JJ, *Adv. Immun.* **50**, 55-85 (1991)
- Fortunato A, Strassmann JE, Santorelli L, Queller DC: **Co-occurrence in nature of different clones of the social amoeba, *Dictyostelium discoideum*.** *Science* 2003, **299**: 1031-1038.

- Foster KR, Fortunato A, Strassmann JE, Queller DC: **The costs and benefits of being a chimera.** *Proceedings of the Royal Society of London, Series B* 2002, **269**: 2357-2362
- Gilbert OM, Foster KR, Mehdiabadi NJ, Strassmann JE, Queller DC: **High relatedness maintains multicellular cooperation in a social amoeba by controlling cheater mutants.** *Proceedings of the National Academy of the Sciences* 2007, **104**: 8931-8917.
- Gilley DC (2001) *Ethology* 107:601-622
- Golstein P, Ojcius DM, Young JD; *Immunol. Rev.* **121**, 29-65 (1991)
- Greenwald I: **LIN-12/Notch signaling: lessons from worms and flies.** *Genes Dev.* **12**, 1751-1762 (1998).
- Grosberg & Strathmann (1998) **One cell, two cell, red cell, blue cell: the persistence of a unicellular stage in multicellular life histories.** *Trends. Ecol. Evol.* **13**, 112-116.
- Hamilton, W. D. *The genetical evolution of social behaviour.* I. *J. Theor. Biol.* **7**, 1-16 (1964).
- Hamilton, W. D. *The genetical evolution of social behaviour.* II. *J. Theor. Biol.* **7**, 17-52 (1964).
- Hammar SP, Mottet NK, *J. Cell Sci.* **8**, 229-236 (1971)
- Kessin, R. H. 2001 *Dictyostelium: evolution, cell biology, and the development of multicellularity.* Cambridge University Press.
- Khare A, Shaulsky G. (2006) **First among equals: competition between genetically identical cells.** *Nat. Rev. Genet.* **7**: 577-583.

- Leach CK, Ashworth JM, Garrod DR: **Cell sorting out during the differentiation of mixtures of metabolically distinct populations of *Dictyostelium discoideum*. *J. Embryol. Exp. Morphol.* 1973, 29, 647-661.**
- Lockshin RA; *Cell Death in Biology and Pathology* (79-122) Chapman & Hall, New York, 1981
- Maeda, Y., and Maeda, M. (1974). **Heterogeneity of the cell population of the cellular slime mold *Dictyostelium discoideum* before aggregation, and its relation to the subsequent locations of the cells. *Exp. Cell Res.* 84, 88–94.**
- Queller, D. C. and Strassmann, J. E. 2003. **Eusociality. *Current Biology* 13: R861-863.**
- Raper K B 1940 **Pseudoplasmodium formation and organisation in *Dictyostelium discoideum*; *J. Elisha Mitchell Sci. Soc.* 56 241–282.**
- Raper KB: *The Dictyostelids*. Princeton University Press (1984).
- Strassmann JE, Zhu Y, Queller DC: **Altruism and social cheating in the social amoeba, *Dictyostelium discoideum*. *Nature* 2000, 408: 965-967.**
- Shaulsky G, Loomis WF: **Cell type regulation in response to expression of ricin A in *Dictyostelium*. *Dev. Biol.* 160, 85-98 (1993).**
- Shaulsky G and Loomis W F 1996 **Initial cell type divergence in *Dictyostelium* is independent of DIF-1; *Dev. Biol.* 174 214–220.**
- Sokal RR and Rohlf FJ, *Biometry* 3rd edition (406-407). W.H. Freeman and Co, New York, 1995.

- Takeuchi, I. (1969). **Establishment of polar organization during slime mold development. In Nucleic Acid Metabolism, Cell Differentiation, and Cancer Growth**, E.V. Cowdry and S. Seno, eds. (New York: Pergamon Press), pp. 297–304.
- Tasaka M, Takeuchi I: **Role of cell sorting in pattern formation in *Dictyostelium discoideum*. *Differentiation* 1981, 18, 191-196.**
- Thompson, C.R.L., and Kay, R.R. (2000). **Cell-fate choice in *Dictyostelium*: intrinsic biases modulate sensitivity to DIF signaling. *Dev. Biol.* 227, 56-64.**
- Thompson, C.R.L., and Kay, R.R. (2000). **The role of DIF-1 signaling in *Dictyostelium* development. *Mol. Cell* 6, 1509-1514.**

2.8 Figures

Figure 1a,b For all lines, doubling time is calculated each day to track population growth. On the days of our assays, the doubling time of cells grown in standard media (HL-5) is consistently shorter than that of cells grown w/o glucose (a) and an acidic environment (b).

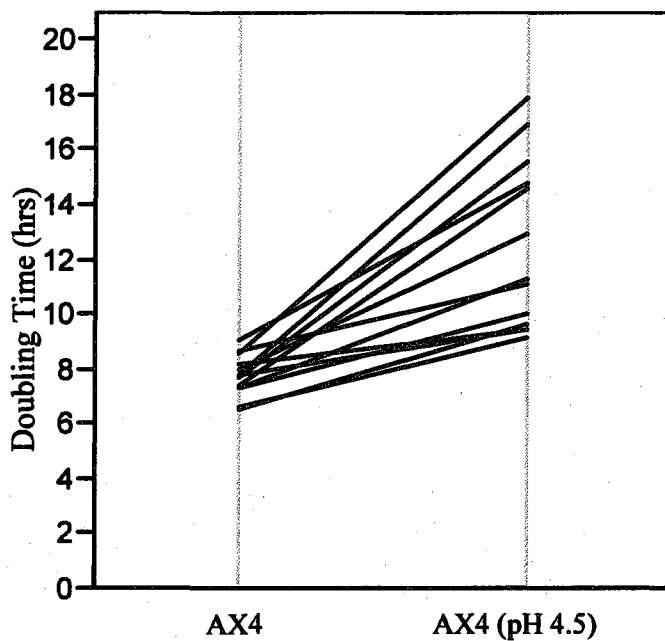
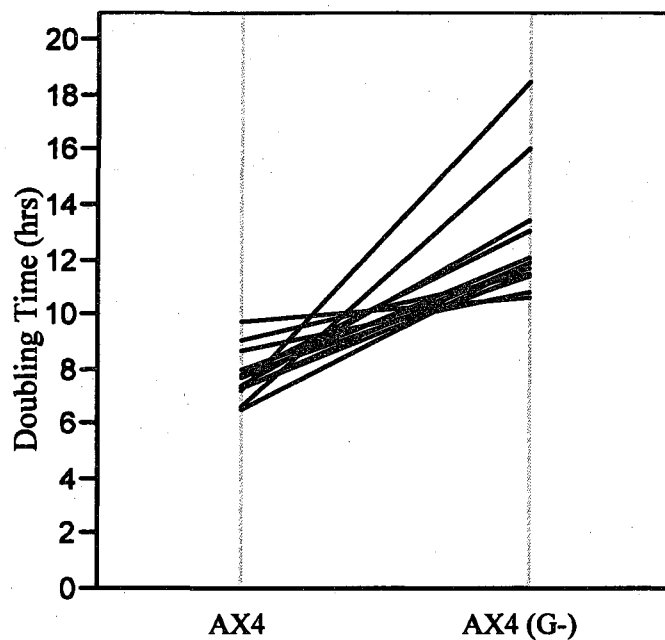


Figure 2 Cells grown without glucose contribute less than their fair share to the spores as compared to stalk. The first two box plots show that there is no effect of labeling (chi-square $p = ns$). The third and fourth show that glucose starved cells (G-) contribute less to spores than their original proportion (0%) in the starting cell mixture whether they were unlabeled (column 3) or labeled (column 4) (chi-square $p < 0.05$). Mixtures began with equal numbers of control AX4 and glucose deprived AX4 cells.

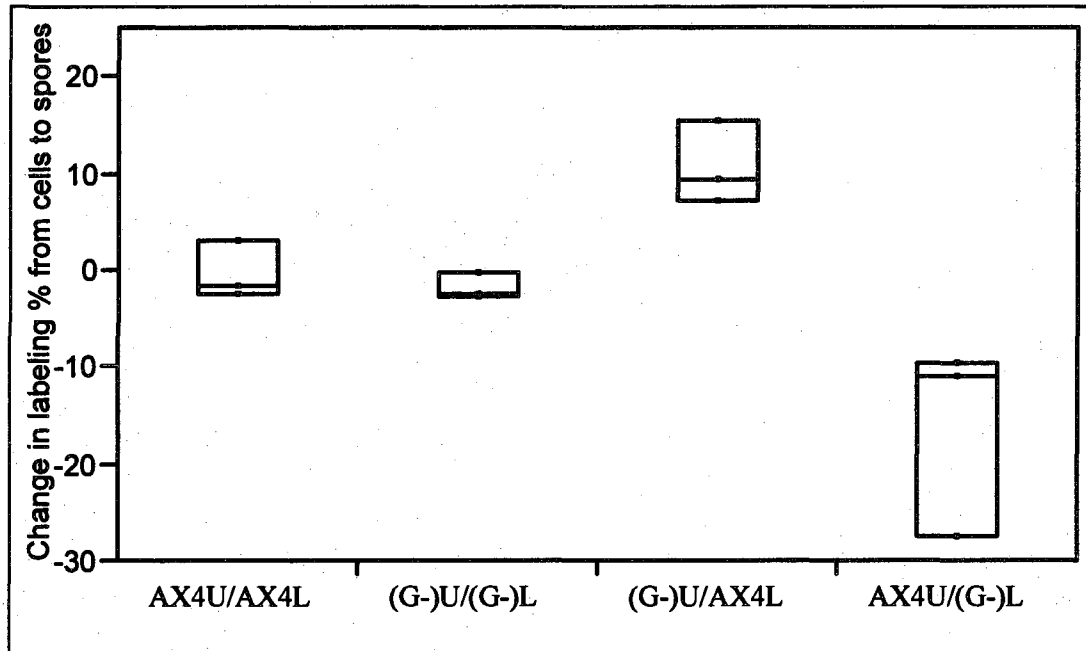


Figure 3 Cells grown in an acidic environment contribute less than their fair share to the spore as compared to stalk. The first two box plots show that there is no effect of labeling (chi-square $p = ns$). The third and fourth show that acid stressed cells (pH) contribute less to spores than their original proportion (0%) in the starting cell mixture whether they were unlabeled (column 3) or labeled (column 4) (chi-square $p < 0.05$). Mixtures began with equal numbers of control AX4 and acid stressed AX4 cells.

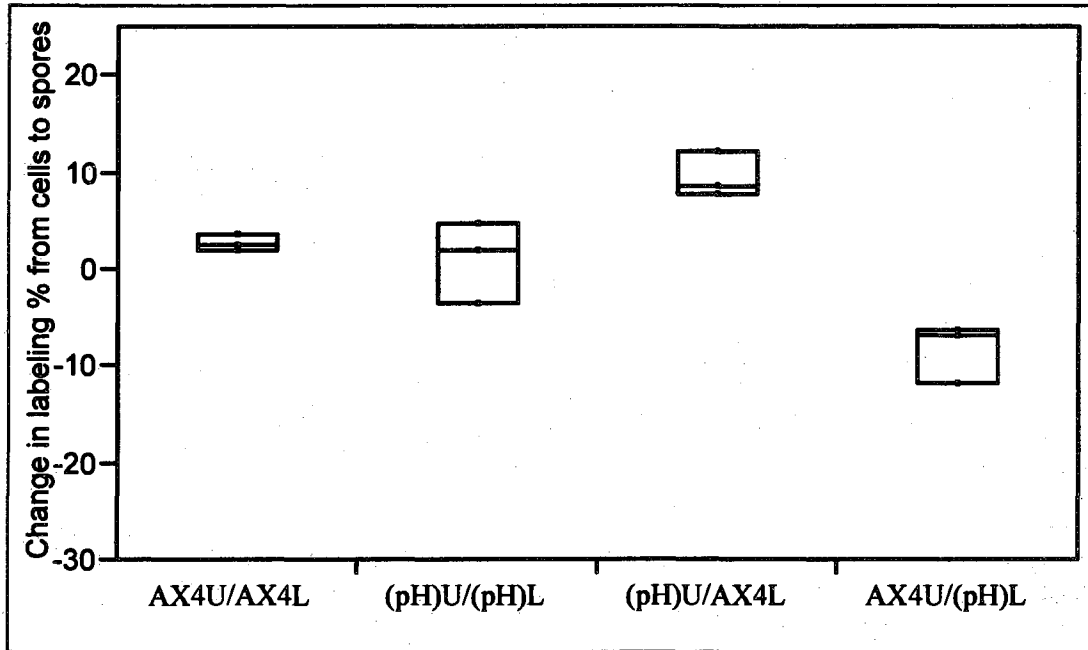


Figure 4 Proportion of spores germinating did not vary with treatment [two way ANOVA, Tukey HSD not significant at $\alpha = 0.05$ for comparisons between AX4 and AX4 (G-) as well as between AX4 and AX4 (pH 4.5)].

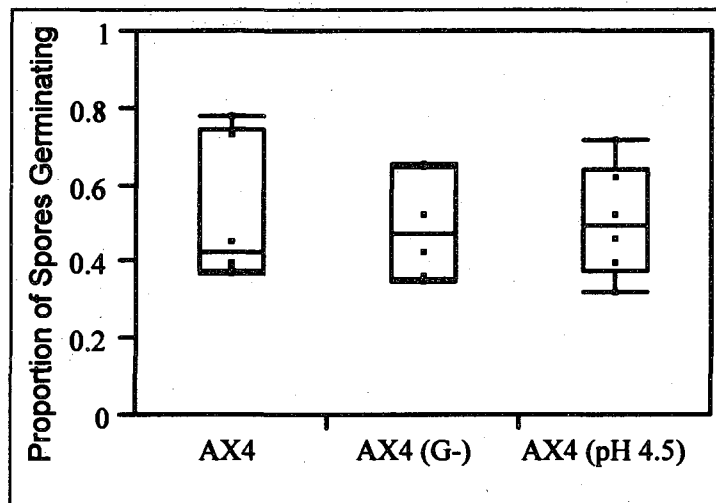


Figure 5a,b New generation cell proliferation after spores hatch is greater in control lines than in cells grown without glucose or in acidic media [two way ANOVA, Tukey HSD significant at $\alpha = 0.05$ for comparisons between AX4 and AX4 (G-) as well as between AX4 and AX4 (pH 4.5)]. Cell proliferation is measured as the area cleared by cells originating from a single spore in a fixed time.

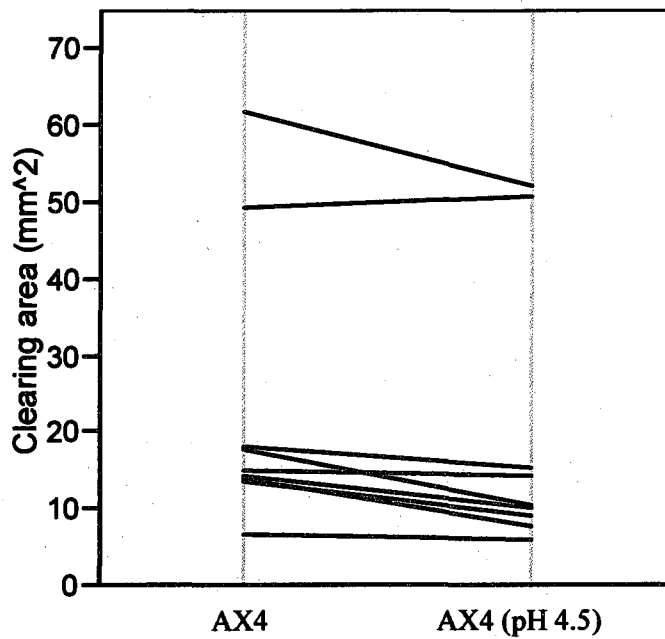
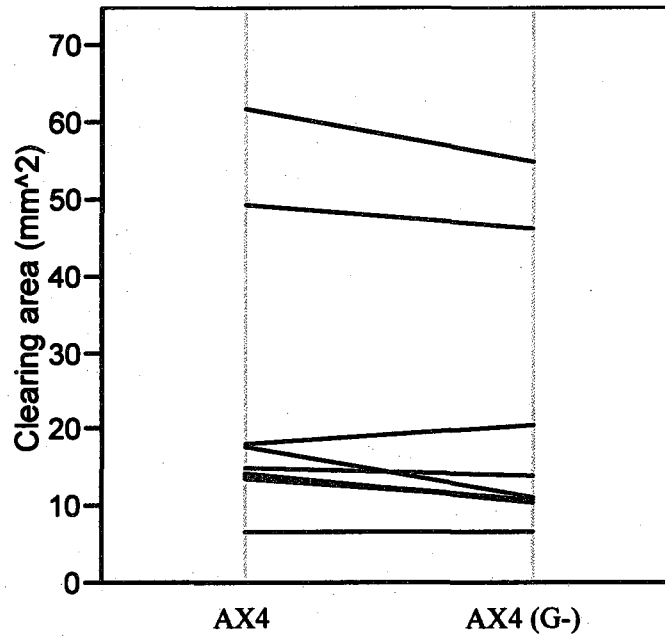


Figure 6 Stressed cells produced fewer spores [two way ANOVA, Tukey HSD significant for comparisons between AX4 and AX4 (G-) as well as between AX4 and AX4 (pH 4.5)]

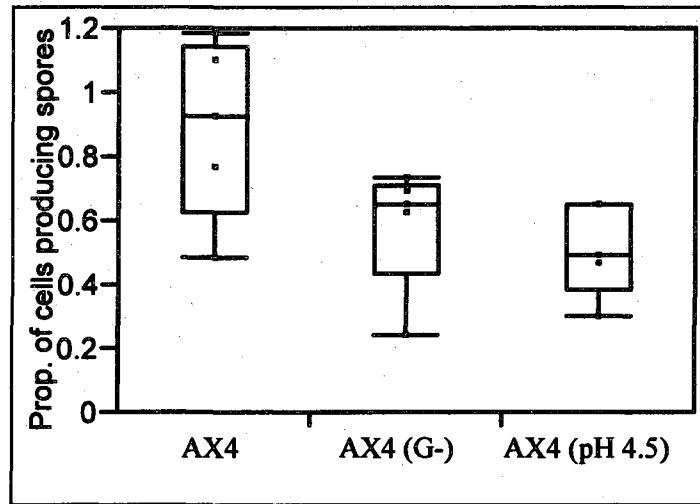


Figure 7 AX4 cells did not show significantly different spore productions when developed with cells of a different condition as compared to when developed alone (two way ANOVA, $df = 5$, $p = 0.719$). The implication is that cells are producing as many spores as they are able regardless of the condition of co-aggregating cells.

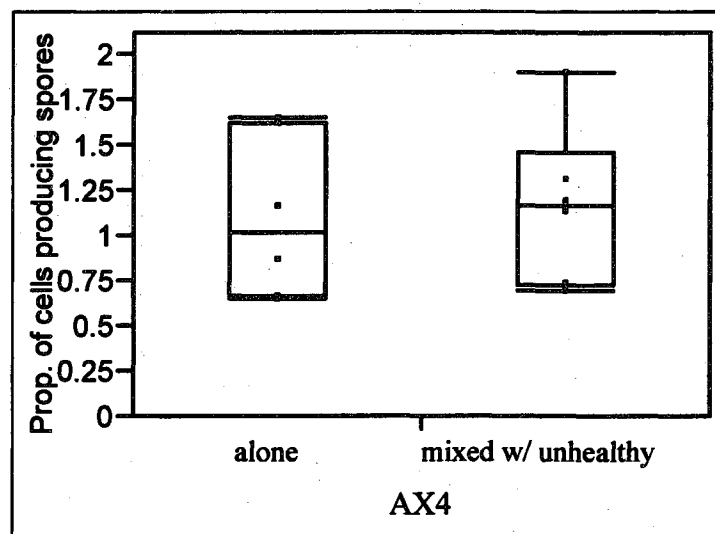


Figure 8 AX4 (G-) cells did not show significantly different spore productions when developed together with cells of a different condition as compared to when developed alone (two way ANOVA, $df = 2$, $p = 0.1383$). The implication is that cells are producing as many spores as they are able regardless of the condition of co-aggregating cells.

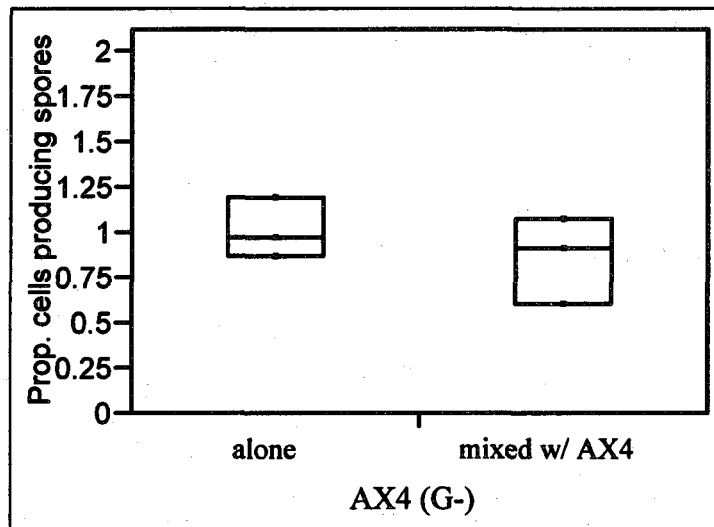


Figure 9 AX4 (pH 4.5) cells did not show significantly different spore productions when developed together with cells of a different condition as compared to when developed alone (two way ANOVA, $df = 2$, $p = 0.8656$). The implication is that cells are producing as many spores as they are able regardless of the condition of co-aggregating cells.

