An invitation to die: initiators of sociality in a social amoeba become selfish spores

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Greater size and strength are common attributes of contest winners. Even in social insects with high cooperation, the right to reproduce falls to the well-fed queens rather than to poorly fed workers. In *Dictyostelium discoideum*, formerly solitary amoebae aggregate when faced with starvation, and some cells die to form a stalk which others ride up to reach a better location to sporulate. The first cells to starve have lower energy reserves than those that starve later, and previous studies have shown that the better-fed cells in a mix tend to form disproportionately more reproductive spores. Therefore, one might expect that the first cells to starve and initiate the social stage should act altruistically and form disproportionately more of the sterile stalk, thereby enticing other better-fed cells into joining the aggregate. This would resemble caste determination in social insects, where altruistic workers are typically fed less than reproductive queens. However, we show that the opposite result holds: the first cells to starve become reproductive spores, presumably by gearing up for competition and outcompeting late starvers to become prespore first. These findings pose the interesting question of why others would join selfish organizers.

Keywords: social evolution; cheating; *Dictyostelium discoideum*; caste determination

1. INTRODUCTION

Cooperation among genes, cells and organisms plays an integral part in many important life-history transitions, such as the evolution of multicellular organisms from single-celled organisms (Maynard Smith & Szathmáry 1995). Control of cheaters is essential in these transitions, since cheating can undermine cooperation. In *Dictyostelium discoideum*, formerly solitary amoebae aggregate when faced with starvation, and roughly 20 per cent of the cells altruistically die to form a stalk that holds aloft a sorus of hardy reproductively capable spores (Shaulsky & Kessin 2007). The stalk is thought to aid in the dispersal of these spores to more favourable environments by water and passing invertebrates (Bonner 1982; Huss 1989). In *D. discoideum*, multiple genetically distinct clones can aggregate to form chimeras, where one clone may cheat and form

disproportionately more of the spores while forcing the other clone to form more than its share of the stalk (Strassmann *et al.* 2000). Since the first cells to starve initiate aggregation into the social stage (Jaiswal *et al.* 2003), it would be interesting to know the fate of these cells. In social insects, better-fed larvae become reproductive queens, and previous work in *D. discoideum* has shown that cells fed glucose are more likely to form spores over cells denied glucose (Leach *et al.* 1973; Thompson & Kay 2000). Therefore, one might expect the first cells to starve and initiate sociality to act altruistically. By forming the stalk that supports the spores produced by better-fed latecomers to the aggregate, they would provide incentive for other cells to join the aggregate. On the other hand, if the first cells to starve were to cheat and preferentially form spores, this could deter other cells from joining the aggregate. Work in the 1980s showed that age is a more important factor than size in determining who supersedes an absent queen in the paper wasps *Polistes exclamans* and *Polistes instabilis*, where older females are more dominant (Strassmann & Meyer 1983; Hughes & Strassmann 1988). Similarly, *D. discoideum* cells that starve early might be able to use this time to gear up for competition to become prespore first and form disproportionately more spores than cells that starve later. Alternatively, the cells that starve first might be equally likely to form spores or stalk cells.

2. MATERIAL AND METHODS

We examined the reproductive fate of the first cells to starve in the axenically grown wild-type laboratory strain AX4, and separately in the bacterially grown wild clone NC75.2 (see electronic supplementary material). In both cases, we split the cells into a prestarved and newly starved treatment and prepared 1 : 1 mixes of the two on nutrient-free substrates, using fluorescent labelling of one treatment to differentiate the two treatments. After the cells had developed for at least two days, we collected the spores and determined the reproductive fate of the prestarved cells by calculating the per cent change of the labelled cells in each mix ((% labelled spores – % labelled cells)/% labelled cells × 100).

(a) Laboratory strain AX4

We raised AX4 and green fluorescent protein-labelled AX4 (AX4-gfp) in shaking nutrient broth at 22°C. While the newly starved treatment cells remained shaking in nutrient broth, we washed the prestarved cells free of nutrients, resuspended them in phosphate buffer and allowed them to shake for 4 h. After this time, we washed the newly starved treatment cells free of nutrients. We prepared 1 : 1 mixes of two reciprocal experimental mixes (prestarved labelled AX4 with newly starved AX4, and newly starved labelled AX4 with prestarved AX4), a newly starved labelling control (newly starved labelled AX4 with newly starved AX4) and a prestarved labelling control (prestarved labelled AX4 w/prestarved AX4), and plated cells of each mix onto nutrient-free substrates.

(b) Wild clone NC75.2

To examine the reproductive fate of the first cells to starve in a more natural scenario, we used the wild clone NC75.2 grown on nutrient plates with *Escherichia coli* at 22°C. We used 5 µM Cell Tracker Green CMFDA to fluorescently label cells of the wild clone NC75.2 that we collected during exponential growth and washed free of nutrients. We allowed the cells to prestarve by developing on a nutrient-free substrate for 1 h before adding unlabelled newly starved NC75.2 cells on top. To ensure there were no negative effects of the labelling process, we prepared a labelling control of labelled and unlabelled NC75.2.

3. RESULTS

Prestarved cells were over-represented in the spores when mixed 1 : 1 with newly starved clonemates (figures 1a,b and 2a). In the AX4 experimental

Figure 1. Box plot showing that within AX4, the first cells to starve cheat. Unless indicated as prestarved, AX4 refers to newly starved AX4. AX4 cells prestarved for 4 h, whether labelled, *a*, or unlabelled, *b*, increased in frequency from the cell to the spore stage when mixed with newly starved cells. Labelling controls, *c*, showed no change. All differences between treatments, except for between the two controls are significant (Tukey–Kramer, $p < 0.05$, $n = 5$).

Figure 2. Box plot showing that within NC75.2, the first cells to starve cheat. Labelled NC75.2 that was prestarved for an hour increased in frequency from the cell to spore stage when mixed with unlabelled newly starved cells, *a*. This was a significantly higher increase than in the labelling control, *b*, with no change (paired-samples t -test, $t_2 = -3.18$, $p < 0.05$).

mixes, the average per cent change in labelled AX4 was significantly higher than in the controls when labelled cells were prestarved (14.70%) and significantly lower than in the controls when unlabelled cells were prestarved (–22.88%) (Tukey–Kramer, $p < 0.05$, $n = 5$). The average per cent change in labelled AX4 was not significantly different between the newly starved (–1.09%) and prestarved controls (–2.09%) (figure 1*c*). In the NC75.2 experimental mix, where labelled NC75.2 was prestarved, the average per cent change of labelled NC75.2 was significantly higher (22.94%) than in the labelling control (0.05%)

(paired-samples t -test, $t_2 = -3.18$, $p < 0.05$). We observed no labelling effects on cheating in either experiment. These results indicate that the first cells to starve are over-represented in the selfish spores and under-represented in the altruistic stalk cells.

4. DISCUSSION

In our experimental mixes, we observed large increases in the percentage of prestarved cells, indicating that early starvers cheat by forming more than their fair share of the spores, while forcing late starvers to produce disproportionately more sterile stalk cells (see electronic supplementary material). Roughly 20 per cent of the cells in an aggregate form the stalk, while 80 per cent form spores. If prestarved cells formed no stalk cells in 1 : 1 mixes with newly starved cells, we would see a 25 per cent increase in prestarved cells among the spores. We observed average increases in prestarved cells ranging from over half this maximum increase to almost the full amount, indicating that entering fruiting body development first is potentially a viable strategy for wild clones to increase their fitness at a cost to another clone.

Studies using wild-type cells showed that cells fed glucose preferentially form prespore cells, and then spores, over cells denied glucose (Leach *et al.* 1973; Thompson & Kay 2000). One might expect prestarved cells to behave like the cells denied glucose and form stalk. Alternatively, starving first could provide an opportunity to gear up for competition with cells that starve later. Loomis (1993) proposed that there is a race among cells entering development to become prespore first and Kay & Thompson (2001) showed that prespore cells, which generally form spores, produce a stalk differentiation inducing factor (DIF), which causes other cells to form stalk. Prestalk cells are also more sensitive to DIF than prespore cells (Thompson & Kay 2000).

Our results extend the finding that a single initiation-competent cell becomes a spore when mixed with mutants that could not initiate aggregation

(Huang *et al.* 1997), by generalizing it to wild-type cells, and showing that it is not an idiosyncratic effect of that mutation. Shaulsky & Kessin (2007) suggested that developing quickly might be a way to cheat, although a number of mutants with this characteristic do not. The *rblA*-null mutant develops more quickly than wild-type, but loses in chimeras with wild-type because of an extreme sensitivity to DIF also conferred by the mutation (MacWilliams *et al.* 2006). The possible pleiotropic effects of mutations may make it unsuitable to compare developmental growth rate and cheating ability in mutants with that of the treated wild-type or wild lines in our experiments.

In wild-type lines, glucose-fed cells start developing before cells denied glucose (Inouye & Takeuchi 1982). Our prestarved NC75.2 cells clearly had an hour head start on development over the newly starved cells they later aggregated with, and AX4 prestarved cells also aggregated and developed faster than newly starved cells (electronic supplementary material, figure S1). Perhaps, the precocious development of prestarved cells gives them a head start in the race to become prespore first and allows them to produce DIF, like glucose-fed prespore cells, before the cells that starve later can, causing late starvers to form stalk.

The success of initiators raises the question of why there is not a selective race to start fruiting body development earlier and earlier. Relatedness is high in nature (Gilbert *et al.* 2007), so cells are likely to form clonal fruiting bodies where there is no fitness advantage to starving first. However, there is an advantage to consuming all available food and dividing as much as possible before entering development. When competing with clones that starve earlier, the magnitude of this benefit depends on how much food is left to consume. There is likely to be relatively little, considering that one clone already sensed starvation. In any case, late starvers would have higher fitness in clonal fruiting bodies than in chimeras with early starvers, where they are cheated into forming disproportionately more stalk. If the time between the onsets of starvation is large, the two clones are unlikely to form chimeras, as we saw almost complete segregation of newly starved NC75.2 and 4 h prestarved NC75.2 (data not shown).

Owing to high relatedness (Gilbert *et al.* 2007), the risk of being cheated in nature is relatively low, but there are also good reasons to join non-clonemates despite the possibility of being cheated. First, entering the social cycle is necessary to produce drought-tolerant spores, since *D. discoideum* cannot form hardy unicellular microcysts (Bonner 1982; Raper 1984). Second, cells may operate under a veil of ignorance, having little information on how many surrounding cells precede or follow them in starvation and their likelihood of becoming a spore. Third, joining a chimera can give size advantages that accrue to a larger multicellular individual. These include longer migration during the motile slug stage (Foster *et al.* 2002) and greater fruiting body height, which may increase dispersal success and outweigh the potential cost of victimization.

Our findings also show that the advantage of starving earlier outweighs disadvantages owing to nutrition. Thus, caste determination in *D. discoideum* is not like that of worker and queen social insects. It is more like

the ascension to queenship in paper wasp colonies by older but smaller workers (Strassmann & Meyer 1983; Hughes & Strassmann 1988).

This material is based upon work supported by the National Science Foundation under grants EF-0328455, DEB-0816690 and EF-DEB-0918931. We thank editor Fiona Pring and the referees for their useful comments.

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