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Concurrent coevolution of intra-organismal cheaters and resisters

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1 Title: Concurrent co-evolution of intra-organismal cheaters and resisters

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Abstract

The evolution of multicellularity is a major transition that is not yet fully understood. Specifically, we do not know if there are any mechanisms by which multicellularity can be maintained without a single cell bottleneck or other relatedness enhancing mechanisms. Under low relatedness, cheaters can evolve that benefit from the altruistic behaviour of others without themselves sacrificing. If these are obligate cheaters, incapable of co-operating, their spread can lead to the demise of multicellularity. One possibility, however, is that co-operators can evolve resistance to cheaters. We tested this idea in a facultatively multicellular social amoeba, *Dictyostelium discoideum*. This amoeba usually exists as a single cell but, when stressed, thousands of cells aggregate to form a multicellular organism in which some of the cells sacrifice for the good of others. We used lineages that had undergone experimental evolution at very low relatedness, during which time obligate cheaters evolved. Unlike earlier experiments, which found resistance to cheaters that were prevented from evolving, we competed cheaters and non-cheaters that evolved together, and cheaters with their ancestors. We found that non-cheaters can evolve resistance to cheating before cheating sweeps through the population and multicellularity is lost. Our results provide insight into cheater-resister co-evolutionary dynamics, in turn providing experimental evidence for the maintenance of at least a simple form of multicellularity by means other than high relatedness.

Keywords: major transition, multicellularity, altruism, cooperation, cheaters, experimental evolution.

Introduction

Multicellularity

Perhaps the most interesting moments in the history of life are the great transformations in the unit of individuality, when what were previously self-sufficient, functioning individuals, become integrated into a collective, no longer capable of replicating independently (Maynard-Smith and Szathmáry 1995). They are interesting in large part because of the questions they raise about conflict. In order for a higher level of biological organization to form, conflict must be controlled at lower levels. Consider, for example, the origin of multicellularity, one of the six widely recognized major transitions (Bourke 2011). Multicellularity requires anywhere from a few to many millions of cells to sacrifice for the good of only a minority. Why do the cells in our hands, hearts, and brains sacrifice their own reproduction so that our gametes can be passed on?

Inclusive fitness provides one answer (Hamilton 1964a;b). With each generation the organism passes through a single celled bottleneck (i.e. the zygote), meaning that all of the cells within the organism are clonally related. Their relatedness is one ($r = 1$), so the genetic basis for conflict is effectively eliminated. In addition, any cheater mutation that gets a cell into the germ-line will be limited to one round of cheating, because in the following generation it will be found in a multicellular organism consisting entirely of its clones (Queller 2000). Thus, inclusive fitness explains how multicellularity can be evolutionarily stable and may also explain the prevalence of single-cell bottlenecks.

However, questions remain. First, there are alternative explanations for the prevalence of single-cell bottlenecks; they might serve to purge deleterious mutations

(Grosberg and Strathmann 1998) or they might be necessary for complex development (Wolpert and Száthmáry 2002). The existence of single-cell bottlenecks therefore cannot be taken as strong evidence for the importance of conflict reduction. Second, although most examples of multicellularity have their origin in clonality, there are a few exceptions, like the social amoeba, *Dictyostelium discoideum*. If multicellularity did not originate in clonality, what level of relatedness would be required amongst the cells for multicellularity to be stable? The examples provided by social insects show that extremely cooperative entities (colonies) can be stable without clonal relatedness, in part through coercion or policing (Ratnieks and Wenseleers 2007), a co-evolutionary response of other parties to the evolution of cheating. Are there other forces like this at play that could promote and maintain multicellularity? For example, could non-cheaters evolve resistance to cheating? Buss (1987) argued that many aspects of multicellular development evolved from such an interplay between cellular cheaters and resisters. Some of these scenarios are implausible under high relatedness (Queller 2000), though others, such as control of cell division rates, might not be (Michod 1997). But if relatedness were low in the evolution of multicellularity, such co-evolutionary responses to cheaters might be required for multicellularity to be maintained. Unfortunately, these questions are difficult to explore experimentally. Most multicellular organisms obligately pass through a single-cell bottleneck, such that their intra-organismal relatedness cannot be experimentally manipulated. Since *D. discoideum* becomes multicellular by aggregation, it is a great system in which to explore what is mostly the path not taken.

Dictyostelium discoideum

90 *D. discoideum* is usually a unicellular amoeba that lives in soil and moist leaf
91 litter, feeding on bacteria. However, when starved, the amoebas aggregate, and form a
92 multicellular slug, which migrates to a new location, at which point about 20% of the
93 cells sacrifice any future reproduction to form a dead cellulose-reinforced stalk. The
94 remaining 80% swarm up this stalk, becoming spores, and forming the sorus (the
95 collection of spores at the top of the fruiting body), which contains thousands of spores
96 (Jack *et al.* 2011). The stalk facilitates the dispersal of spores by animal vectors to a new
97 location (smith *et al.* 2014) where they hatch into single-cell amoebas. Spores within
98 natural fruiting bodies have high relatedness (Gilbert *et al.* 2007). This is probably
99 largely due to the isolation of founder cells, but also partly due to weak kin recognition
100 systems (Benabentos *et al.* 2009; Hirose *et al.* 2011; Gilbert *et al.* 2012). High relatedness
101 is not necessary for aggregation – different clones mix readily in lab experiments
102 (Strassmann *et al.* 2000) – but the high relatedness in the field explains why
103 multicellularity can be stable even though the fruiting bodies form by aggregation
104 (Fortunato *et al.* 2003; Ostrowski *et al.* 2008). This system has been enormously useful
105 for empirical social evolution work, particularly with regard to the origin of
106 multicellularity. Its utility stems from a variety of factors, the most important being that,
107 unlike organisms with single-cell bottlenecks, *D. discoideum*'s intra-organismal
108 relatedness can be manipulated. An experimenter can decide which cells aggregate to
109 form a slug, thus changing the degree of relatedness of the aggregating cells. Indeed, an
110 important prediction of the major transitions view of evolution – that multicellularity is
111 stabilized by self-limitation due to high intra-organismal relatedness – has been tested
112 using *D. discoideum* (Kuzdzal-Fick *et al.* 2011).

Cheating of Multicellularity in Dictyostelium

Kuzdzal-Fick *et al.* (2011), starting with a single clone, artificially maintained low relatedness in *D. discoideum* for 31 rounds of vegetative growth, starvation and spore formation. Low relatedness, maintained by starting each new generation with a random mixture of 10^6 spores, allowed cheaters that appear by mutation to be favoured by selection. These cheaters cheat by increasing their representation in the sorus, while contributing little or nothing to the stalk.

Further, some of these evolved cheaters were obligate cheaters, which cannot produce fruiting bodies on their own. Obligate cheaters, unlike facultative cheaters, do not modulate their cheating based on their partners (Travisano and Velicer 2004; Ghoul *et al.* 2014). Here, cheating entails not sacrificing to form the stalk. In mixtures this works because the other, non-cheating clone forms the stalk. But when alone, an obligate refusal to form stalk means that no spores form either (Ennis *et al.* 2000; Gilbert *et al.* 2007), so the organism has no fitness. The importance of this distinction is that co-operation can persist in the presence of facultative cheaters, but obligate non-fruiting, if it sweeps through the population, eliminates co-operation. Obligate non-fruiters also have an added experimental value, because the cheaters can be readily identified when plated out clonally, as they fail to fruit, simply forming a small group of cells.

Even though mutation rates to obligate non-fruiting cheaters are known to be low (Hall *et al.* 2013), cheaters readily rise to high frequencies when intra-organismal low relatedness provides them with fruiting victims to exploit (Kuzdzal-Fick *et al.* 2011). At high relatedness, such as occurs in natural fruiting bodies, these mutants do poorly

(Gilbert *et al.* 2007). This raises a question about the evolution of multicellularity. If relatedness were not high, would anything prevent cheating from sweeping through the population? Several mechanisms could be involved (Strassmann and Queller 2011), one of which is that co-operators could evolve resistance to cheating. Khare *et al.* (2009) demonstrated that resistance to cheating can be selected for when the cheaters are held constant (not evolving). They presented *D. discoideum* populations with a cheater for four cycles of selection. They showed that the repeated presence of the cheater selected for resistance to cheating. This experiment suggests that it may be possible for co-operators to evolve resistance to cheating. Hollis (2012) tested co-evolving populations of *Dictyostelium* cheaters and non-cheaters, and populations of evolving non-cheaters against non-evolving cheaters, and only found evidence for the evolution of resistance when the cheaters were not allowed to evolve. Therefore, it has yet to be demonstrated that a co-evolutionary response to cheating can evolve before cheating sweeps through population and multicellularity is lost. Ideally we would like to know if resistance evolves in real populations, with the cheaters and non-cheaters co-evolving in real time. Our experiments explore this question.

Resistance experiment

Our experiments test whether *D. discoideum* can evolve resistance to cheating while cheaters are evolving, before the obligate cheating phenotype sweeps through the population. We used lineages from the Kuzdzal-Fick (2011) experiment, which underwent experimental evolution at low relatedness and evolved cheating. Non-fruiting clones – potential obligate cheaters – increased in the experiment and three of four tested

against the (fruiting) ancestor were confirmed to be cheaters (Kuzdzal-Fick (2011)). We first confirmed that this ability to cheat the ancestor held for much larger numbers of non-fruiting clones. Then, to test whether resistance had also evolved, we tested the non-fruiters against fruiting clones isolated from their own selection lines. If resistance has not evolved, we would expect to find the same proportion of non-fruiters in the sori of both mixtures (evolved non-fruiters with ancestors and evolved non-fruiters with evolved fruiters). If the evolved fruiters have evolved resistance to cheating we would expect them to be better than the ancestors at keeping the non-fruiter out of the sorus.

Our results showed that resistance to cheating did evolve. Because we worked with clones from populations that evolved from a single clone, this means that resistance evolved after the obligate cheaters emerged but before they swept through the population. This has implications for both our understanding of the evolutionary dynamics of cheating and resistance (e.g. co-operators can evolve resistance to cheating in real time), and our understanding of the evolution of multicellularity (e.g. there are mechanisms other than self-limitation that can stabilise simple multicellularity).

Materials and Methods

Amoebas

We used the ancestor and 4 experimental lines from the Kuzdzal-Fick *et al.* 2011 study. The experimental lines had gone through thirty-one rounds of fruiting, with each round initiated by spreading a million cells across a new plate, so any new cheater mutation would be well-mixed among victims (Kuzdzal-Fick *et al.* (2011)). All four of

these experimental lines had a reported frequency of non-fruiting mutants of 50% or more, which we verified with an initial plating of all the lines.

All lines had been frozen as spores in KK2 Buffer (per liter: 2.25 g KH_2HPO_4 , 0.67 g K_2HPO_4) with 25% glycerol at -80 degrees Celsius, as described in the Supporting Online Material for Kuzdzal-Fick *et al* (2011). For all frozen samples, we thawed them gently (at room temperature), diluted quickly, counted spores using a haemocytometer, and then plated 50 spores each onto 10 mL SM/5 plates with 200 μL of *Klebsiella pneumoniae* in KK2 (OD600 1.5). Plated spores or plated amoebas took between 76-80 hours to form fruiting bodies. After fruiting, we allowed one week before collecting fruiting bodies. Details of culturing can be found in Kuzdzal-Fick *et al.* (2011).

Population Experiment

The purpose of the population experiment was to determine whether or not there was resistance to cheating in the evolved populations. We achieved this by competing populations of putatively cheating evolved non-fruiters against both the ancestor (which is a fruiter) and populations of evolved fruiters from the same line. Here population is defined as a mixture of 25 separate clonal plaques of each type. If non-fruiters do cheat the ancestor as expected, resistance to cheating will be established if they cheat the evolved fruiters less or not at all. If the non-fruiter cheats both the ancestor and the evolved fruiter equally, we would expect to see the same number of non-fruiters in the sori for both mixtures. This experiment is illustrated diagrammatically in Figure 1.

In this experiment, for each line (Kuzdzal-Fick lines: 12, 16, 21, and 24), we initially plated spores at low density (50 spores per plate, 10 plates for each line) and

205 allowed them to fruit. For each line we then picked and mixed 25 evolved non-fruiters
206 and, separately, 25 evolved fruiters. We did this by categorising colonies (by phenotype)
207 as either fruiters or non-fruiters, and then for each mixture, picking the leading edge of a
208 colony with a loop, adding the cells to a 1.5 mL micro-centrifuge tube with 1mL HL5
209 liquid medium (35.5g per liter), sterilising the loop, and repeating 24 times. Each mixture
210 of 25 clones was created this way for all four lines. We also picked and mixed 4-5
211 ancestor colonies in the same fashion.

212 The cell mixtures were cultured in 10 mL HL5 with PSV antibiotic for 2-3 days in
213 10mL tissue culture plates to allow growth of enough cells to plate at high density. We
214 split the cultures every 24 hours to keep the cells from overcrowding the plates. This was
215 achieved by pipetting the solution up and down with an electronic pipette to lift the
216 amoebas from the bottom of the tissue culture plate, transferring 5mL of the solution to a
217 fresh tissue culture plate, and then adding 5mL of fresh HL5 with PSV antibiotic to each
218 plate.

219 We then washed the cells three times using HL5 with no antibiotic, so that we
220 could plate them on SM/5 plates with bacteria as a food source without killing the
221 bacteria. We counted the cells, and then plated them with *K. pneumoniae* in the following
222 ratios: 25% evolved non-fruiters with 75% evolved fruiters, and 25% evolved non-
223 fruiters with 75% ancestor. We also plated each culture on its own (evolved non-fruiter
224 alone, evolved fruiter alone, and ancestor alone), as a control to ensure that all the cells
225 grew up on plates successfully. We plated 2×10^5 cells on each plate in order to allow
226 thorough mixing of the different clones, and allowed 76-80 hours for the amoebas to
227 fruit, plus 7 days for the fruiting bodies to be more easily harvestable.

Next we pooled spores from 4 fruiting bodies from each plate (evolved non-fruiter with ancestor and evolved non-fruiter with evolved fruiter), and plated 1000 spores from each plate onto 20 SM/5 plates with *K. pneumoniae* (20 plates per experiment mixture, 2 mixtures per line, 4 lines, 160 plates). We diluted the spores across so many plates because we wanted to see individual plaques as fruiterers or non-fruiterers (our measure of non-cheaters and cheaters). We allowed 76-80 hours for these to fruit, and then scored each colony as either a fruiter or a non-fruiter. We counted the total number of fruiterers and non-fruiterers for each experimental mixture, and used this to determine the proportion of non-fruiterers. This served as our measure of the degree of cheating, because it measured how many of the non-fruiterers ended up as spore rather than stalk cells.

Individual Experiment

The purpose of the individual experiment was to confirm the results of the population experiment at the individual level by looking for resistance to cheating in individual evolved fruiter clones (rather than evolved fruiter populations), and to look for variation among individual evolved fruiter clones. If individual evolved fruiter clones have resistance, we would expect fewer non-fruiterers to get into the sori in mixtures of evolved non-fruiterers and evolved fruiterers than in mixtures of evolved non-fruiterers and ancestors. However, if not all evolved fruiterers have resistance, or if there are different kinds of resistance in the population, we would expect some mixtures of non-fruiterers and

fruiters to yield the same number of non-fruiters in the sori as in mixtures with ancestors.
The individual experiment is depicted diagrammatically in Figure 2.

In the individual experiment we used the three lines that most clearly appeared to have evolved resistance in the population experiment: 12, 16, and 21. We plated these lines and the ancestor from the freezer at low density (50 spores on each of 5 plates for the three lines). We allowed 76-80 hours for the amoebas to fruit, and then picked two fruiter colonies and two non-fruiter colonies for each line. We cultured each clone separately in HL5 + PSV as described previously (thus, four cultures for each line: non-fruiter 1, non-fruiter 2, fruiter 1, and fruiter 2). We cultured ancestors in the same fashion.

We cultured the clones for three days. On the second day of culture, we split the 10 mL of culture into two plates. After three days of culture, we washed the cells of antibiotic by centrifuging the cultures in 15 mL centrifuge tubes and adding fresh HL5 with no antibiotic three times. We then diluted the cells to 1×10^7 cells mL^{-1} . We plated six experimental mixtures from each line, testing each of the evolved non-fruiters against both the evolved fruiters from its own line and the ancestor: Evolved Non-Fruiter 1 + Evolved Fruiter 1, Evolved Non-Fruiter 1 + Evolved Fruiter 2, Evolved Non-Fruiter 2 + Evolved Fruiter 1, Evolved Non-Fruiter 2 + Evolved Fruiter 2, Evolved Non-Fruiter 1 + Ancestor, and Evolved Non-Fruiter 2 + Ancestor. The mixtures were a 75:25 ratio, with the non-fruiter always making up 75% of the mixture. We also plated each clone on its own as a control to ensure that each clone was still healthy after being in liquid culture (and that there was no fruiter/non-fruiter contamination). We allowed ten days for the fruiting bodies to reach the stage of having harvestable sori.

We then harvested all of the fruiting bodies for each mixture using a loop. We diluted the spores from each mixture to 1000 spores in 4 mL of *K. pneumoniae* in KK2 (OD600 1.5). For each mixture, we plated 200 μ L of solution onto each of 20 plates, with the aim of having roughly 50 spores per plate. We allowed three days for the spores to hatch, develop, and reach the fruiting stages so we could score them as fruiterers or nonfruiterers.

Statistical Analyses

We conducted all statistical analyses using R version 2.15.2 (released October 2012). We arcsine-square root transformed all proportion data (number of evolved non-fruiter spores out of total spores in the sorus) to compensate for skew created by the 0 and 1 boundaries of proportional data. We analysed the data from the population experiment with a paired t-test (95% confidence level). We analysed results from the individual experiment using a Welch two sample t-test for samples of unequal sizes (95% confidence level), and multiple two-tailed binomial tests (95% confidence level).

For the figures, we plotted relative sporulation efficiency against mixture. Relative sporulation efficiency ratio is the ratio of cheater sporulation efficiency to competitor (ancestor or evolved fruiter) sporulation efficiency. Sporulation efficiency is calculated as fraction of spores in the sorus over fraction of cells in the initial mixture (see Buttery *et al.* 2013).

295

296 **Results**

297 *Population Experiment*

298 The population experiment tested for resistance to cheating in non-cheaters from
299 experimentally evolved populations that contained cheaters. We did this by testing
300 whether the evolved fruiterers did better than the ancestral fruiterers against the evolved
301 cheating non-fruiterers.

302

303 In mixtures with the ancestor, the proportion of non-fruiter increased significantly
304 (two-tailed, one-sample t-test, $t_3=6.75$, $p=0.007$). The proportion of evolved non-fruiter
305 rose from 0.25 to a mean of 0.660 (95% CI: $0.463 < u < 0.832$), with their sporulation
306 efficiency about 7 times that of the ancestor (Figure 3). In contrast, the proportion of
307 evolved non-fruiter did not significantly change in mixtures with the evolved fruiterers
308 (two-tailed, one-sample t-test, $t_3=1.48$, $p=0.235$). The mean proportion of non-fruiter rose
309 from 0.25 to a mean of 0.344 (95% CI: $0.155 < u < 0.563$). A paired t-test rejects the null
310 hypothesis that there is no difference in non-fruiter proportion between evolved fruiter
311 mixtures and ancestor mixtures (Figure 3, $t_3=-8.29$ $p=0.004$), and therefore supports the
312 evolution of resistance.

313

314

315 *Individual Experiment*

316 The individual experiment tested for resistance to cheating in individual evolved
317 fruiter clones.

The proportion of evolved non-fruiterers increased significantly in mixtures with ancestors (two-tailed, one-sample t-test, $t_5=11.04$, $p<0.001$). The mean proportion of non-fruiterers increased from 0.75 to a mean of 0.916 (95% CI: $0.884 < u < 0.943$). The proportion of evolved non-fruiterers in mixtures with evolved fruiterers did not significantly change (two-tailed, one-sample t-test, $t_{11}=-1.09$, $p=0.298$). The mean proportion of non-fruiterers decreased slightly from 0.75 to 0.682 (Figure 4, 95% CI: $0.533 < u < 0.813$). A Welch two sample t-test rejects the null hypothesis that there is no difference in mean proportion between mixtures with ancestors and mixtures with evolved fruiterers ($t_{12,9}=-4.22$, $p=0.001$).

The proportion of non-fruiterers increased significantly in six out of six ancestor mixtures (Figure 5, $p < 0.05$, two-tailed binomial tests, 95% confidence level). However, the tests of non-fruiterers with evolved fruiterers showed much more variation. In two of the evolved fruiter mixtures (16 F1 + NF2, and 21 F1 +NF2), the proportion of non-fruiterers did not change significantly ($p = 0.564$ and $p = 0.143$, respectively, two-tailed binomial tests, 95% confidence level). In four of the evolved fruiter mixtures, the proportion of non-fruiterers went up significantly, and in six of the fruiter mixtures, the proportion of non-fruiterers significantly decreased ($p < 0.05$, two-tailed binomial tests, 95% confidence level).

Discussion

Our results add to a growing body of knowledge regarding the nature and dynamics of cheating and resistance. Numerous studies have explored the nature of

cheating, both in *Dictyostelium* (e.g. Ennis *et al.* 2000; Strassmann *et al.* 2000; Buttery *et al.* 2009; Buttery *et al.* 2013) and across a wide variety of other taxa. For example, cleaner fish cheat by biting their host instead of cleaning (e.g. Bshary and Grutter 2002 for fish), co-operatively scavenging *Pseudomonas* bacteria cheat by not producing costly iron scavenging siderophore molecules (Griffin *et al.* 2004), *Myxococcus* bacteria cheat in cooperative spore formation (Velicer *et al.* 2000), and fork-tailed drongos mimic alarm calls of pied babblers in order to gain access to food (Ridley *et al.* 2007; Flower 2011) (see Ghoul *et al.* 2014 for a review of cheating). Our experiment follows up the demonstration by Kuzdzal-Fick *et al.* (2011) that low relatedness leads to the evolution of obligate cheating, and shows that this cheating is widespread, adding to the stack of empirical evidence that relatedness is important for co-operation and prevents the spread of cheaters. However, factors other than relatedness and kin selection can be important in limiting cheating. This must be the case in between-species interactions where relatedness cannot play a role. Thus, client fish will avoid cheating cleaner fish that bite (Pinto *et al.* 2011) and legumes may shut off resources to nodules that fail to produce nitrogen (Kiers *et al.* 2003). Even within species, cheating is sometimes controlled by evolutionary responses among the cheated. For example in social insects, egg laying by subordinates can be controlled either by dominant queens or through policing by other workers (Queller and Strassmann 1998). Our experiments, which explored the co-evolution of cheaters and resisters in evolving *D. discoideum* lineages, tested whether cheater resistance could play this role in the evolution of multicellularity.

We first confirmed the result of Kuzdzal-Fick *et al.* (2011) that most non-fruiters are obligate cheaters that cheat their ancestor. In the population experiment, the

proportion of non-fruiters in the sorus, on average, increased significantly in ancestor mixtures (Figure 3). In the individual experiment, the proportion of non-fruiters increased significantly in six out of six ancestor mixtures. The latter result brings the total for both studies to nine out of ten.

Our main question is whether this evolution of cheating non-fruiters provoked a co-evolutionary response among the fruiters. Both the population and individual experiments show that fruiting clones that have evolved in the presence of non-fruiters (evolved fruiters) are resistant to the evolved non-fruiters' cheating. In the population experiment, there was no significant change in non-fruiter proportion in mixtures with the evolved fruiter, and in both experiments, evolved fruiters did significantly better than the ancestor when tested with non-fruiters.

A possible alternative explanation for this result is that the 31 extra rounds of adaptation to the lab environment makes both evolved fruiters and non-fruiters better than the ancestor. This seems very unlikely for two reasons. First, better adaptation to the growth environment is not expected to enhance cheating; clones that produce more spores on their own do not typically produce more spores per cell in mixtures (Buttery *et al.* 2009). Second, the clone used should already have been well adapted to the environment. Kuzdzal-Fick *et al.* (2011) used a clone that is descended from strain NC4 collected in the wild in 1933 (Raper 1984), so it had been in the laboratory environment for over 75 years where it has undergone extensive evolution (Bloomfield *et al.* 2008). The experimental evolution took place in SM/5 medium with *Klebsiella aerogenes* on agar plates (Kuzdzal-Fick *et al.* 2011), an environment that would have been commonly encountered in those 75 years. Third, to further guard against possible issues of lab

adaptation, the Kuzdzal-Fick evolution experiment was initiated with a clone taken from a line previously evolved in the exact same experimental evolution conditions for ten rounds of fruiting and about 100 cell generations (Kuzdzal-Fick *et al.* 2011). The only real novelty in the experimental evolution environment was the presence of cheaters due to low relatedness. Fourth, if the lab adaptation hypothesis were true, it would imply the strange result that, leaving aside the obligate cheating trait itself, the fruiterers are consistently evolving more rapidly to the lab than the cheaters are.

A potential test of lab adaptation by the evolved fruiterers would involve competing them against the ancestor, but this would require different and less comparable methods because we could not assess frequencies via incidence of non-fruiting. Moreover, even if it did show that the evolved non-fruiterers did better against the ancestor, that would at most add a dimension to our understanding of the selection (e.g. see Asfahl *et al.* 2015 for an example where the adaptation was non-social). It would not take away the component we have demonstrated – that evolved fruiterers resist cheating of the non-fruiterers and that this advantage would have been in play in the non-fruiter containing environments where they evolved. Resistance cannot be said to be a side effect if it played a demonstrable part in the selection.

Another possible explanation, though not really an alternative one, is that kin recognition and segregation evolved during the course of the experimental evolution so that when fruiterers and non-fruiterers are mixed, they segregate out and the non-fruiterers do poorly on their own. However, we did not see evidence of this. The lawns of fruiting bodies from the mixture experiments were healthy and uniform, without defective fruiting bodies. This is not surprising because, although *D. discoideum* does have some

degree of kin recognition (Ostrowski *et al.* 2008, Benabentos *et al.* 2009; Hirose *et al.* 2011), the resulting segregation is generally rather weak (Gilbert *et al.* 2012). It seems unlikely that our clones would have evolved much stronger segregation in 31 rounds of fruiting than natural clones have evolved over countless generations in the field. However, the issue could bear further investigation, not as an alternative hypothesis, but as one possible mechanism for the evolution of resistance to the non-fruiting cheaters.

In our experiment resistance evolved before obligate cheating could sweep through the population, breaking down multicellularity. Prior experiments showing the evolution of resistance either could not test this because they artificially kept the cheaters from increasing or evolving (Khare *et al.* 2009), or failed to find co-evolution of resistance, perhaps due to experimental design (Hollis 2012). In the latter case, the lack of findings could be due to having only 10 generations of co-evolution, perhaps because the main interest in that experiment was selection for cheating rather than resistance.

Our result offers a potential mechanism by which facultative multicellularity remains stable. The lineages used were kept under artificially maintained low-relatedness, so the experiment demonstrates that in the evolution of facultative multicellularity, if there were no high population-structure to keep relatedness high, there could be another mechanism by which multicellularity is maintained: the evolution of cheater resistance. This might explain why no lineages went extinct in the original experiment, although many were showing lower spore production (Kuzdzal-Fick *et al.* 2011). We cannot exclude the possibility that they would go extinct given enough time, but the evolution of resisters should at least slow the process. And even if extinction would ultimately occur

with near-zero relatedness, a modest degree of relatedness together with resistance evolution might be sufficient to prevent extinction.

The individual experiment also suggests that there may be variation in resistance phenotypes in the population. The proportions of non-fruiters were much more variable in evolved fruiter mixtures than ancestor mixtures. In two of the mixtures no cheating occurred, in four of the mixtures the evolved fruiter was cheated, and in six of the mixtures the evolved-fruiter appeared to cheat the cheater. This raises the possibility that, although the frequency of resistance in the population may be similar across lineages, not all individuals have evolved resistance and there may be different phenotypes for resistance.

These results provoke interesting questions about the nature of resistance. The population experiment supports previous work (Khare *et al.* 2009; Hollis 2012) that showed that resisters can be noble, meaning they do not cheat the cheater, they only prevent it from cheating (Khare *et al.* 2009; Hollis 2012). Though this was true in our experiments on average, in a number of our individual tests, the evolved fruiter was not only resistant, but also ignobly cheated the non-fruiter obligate cheater.

Some cheater genotypes have already been identified, but little is known of the mechanism by which cheating works (Santorelli *et al.* 2013), and nothing is known about resister genetics or mechanisms. One question that remains is how frequency affects the dynamics of co-evolution. In our cheating tests, we tested only one mixture frequency but previous work with both non-fruiter and fruiter cheaters suggests that who cheats does not generally change with frequency (Gilbert *et al.* 2008; Buttery *et al.* 2009). Another open question is the amount of variation in the different cheater/resister

phenotypes and how they interact with each other. The results from the individual experiment suggest that this would be a valuable path to pursue.

These questions are relevant to the broader research programme on co-evolutionary arms races. There has already been some evidence of cheater-resister evolutionary arms races (Ghoul *et al.* 2014). Among many examples, there is an evolutionary arms race between brood parasitic cuckoos and their hosts, in which cuckoos are selected to cheat their hosts through egg mimicry and their hosts are selected to detect the deception (Davies 2000; Spottiswoode and Stevens 2010; Langmore *et al.* 2011; Stoddard and Stevens 2011). Although co-evolution was originally conceived for cases like this that concern interactions between species, the concept has long been extended to within-species reciprocal interactions, such as between the sexes (Arnqvist and Rowe 2002) or between nuclear and cytoplasmic genes (Werren 1987). In our experiment, in only 31 rounds of experimental evolution, non-fruiting and fruiting types evolved in response to each other. These results suggest that a co-evolutionary arms race could occur among cell types in facultatively multicellular organisms. This would be particularly likely for resisters that are “ignoble”, and cheat the cheaters. Pursuing this avenue of research will add to our knowledge of cheating, resistance, and evolutionary arms races more broadly.

Cancer may provide another example of where cheater-resister evolution is important in the context of multicellularity. Cancerous cells can be considered cheaters at the intra-organismal level (Nunney 1999; Bourke 2011; Ghoul *et al.* 2014).

Understanding how non-cheaters can resist cheaters, particularly in a noble way that

maintains co-operation at the organismal level, is a potentially valuable approach for research on cancerous cheats.

Conclusion

Our findings demonstrate that, in *Dictyostelium discoideum*, non-cheaters can evolve resistance to cheaters when both are evolving together, and that they can do so before obligate cheating sweeps through the population and multicellularity is lost. This offers a mechanism by which, even if low relatedness conditions occurred in the evolution of facultative multicellularity, at least a simple form of multicellularity could be stabilised by the evolution of resistance to cheating.

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Population Experiment

Figure 1. A diagrammatic representation of the population experiment. Blue plates are 10 mL SM/5 plates with 200 μ L of *Klebsiella pneumoniae* in KK2 (OD600 1.5). Orange plates are 10mL tissue culture plates containing HL5 with PSV antibiotic. Micro-centrifuge tubes are 1.5 mL.

Figure 2. Diagrammatic representation of the individual experiment. Blue plates are 10 mL SM/5 plates with 200 μ L of *Klebsiella pneumoniae* in KK2 (OD600 1.5). Orange plates are 10mL tissue culture plates containing HL5 with PSV antibiotic. Micro-centrifuge tubes are 1.5 mL.

Figure 3. Populations of evolved fruiter resist cheaters better than ancestors resist cheaters. Plot of cheater sporulation efficiency ratio of non-fruiter in ancestor and evolved fruiter mixtures (n=4). Outliers (1.5 times the inter-quartile range (IQR) greater than the upper quartile or 1.5 times the IQR less than the lower quartile) are shown as points. Y-axis is log scale. The cheater has a higher sporulation efficiency in ancestor mixtures than in evolved fruiter mixtures ($p=0.022$, paired t-test). A relative sporulation efficiency of 1 would be no cheating, and higher values suggest cheating has occurred (dotted line at $y=1$ reference).

Figure 4. Individual clones of evolved fruiter resist cheaters better than ancestors resist cheaters. Plot of cheater sporulation efficiency ratio of non-fruiter in ancestor and evolved fruiter mixtures of individual clones (n=6 for ancestor mixtures, n=12 for evolved fruiter mixtures). Outliers (1.5 times the IQR greater than the upper quartile or 1.5 times the IQR less than the lower quartile) are shown as points. Y-axis is log scale. The cheater has a higher sporulation efficiency in ancestor mixtures than in evolved fruiter mixtures (p=0.012, Welch two sample t-test). A relative sporulation efficiency of 1 would be no cheating, and higher values suggest cheating has occurred (dotted line at y=1 reference).

Figure 5. In the individual experiment, cheaters cheat the ancestor consistently but have variable results with the evolved fruiterers. Proportion non-fruiter in the sori resulting from mixtures of the evolved non-fruiter with either the evolved fruiterers or the ancestors from three lineages (12, 16, 21). A=ancestor, F=fruiter, NF=Non-fruiter. Error bars are 95% confidence intervals. Initial proportion non-fruiter, 0.75, shown as dotted for reference. Values above 0.75 represent cheating.