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Concurrent coevolution of intra-organismal cheaters and resisters

S R. Levin

D A. Brock

David C. Queller
Washington University in St Louis, queller@WUSTL.EDU

Joan E. Strassmann
Washington University in St Louis, strassmann@WUSTL.EDU

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

2   Authors: Samuel R. Levin[1, 2, *], Debra A. Brock[2], David C. Queller[2], and Joan E.

3   Strassmann[2]

4   1. Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS,

5   United Kingdom. 2. Department of Biology, Washington University in St. Louis, St.

6   Louis, MO 63130, USA.

7   Short running title: Co-evolution of cheaters and resisters.

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9   *Corresponding author: Samuel R. Levin, Raikes Barn, Hulme End, Buxton, Derbyshire

10   SK17 0HJ. Telephone: +44 7943334161. E-mail: samuel.r.levin@gmail.com

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21 **Abstract**

22   The evolution of multicellularity is a major transition that is not yet fully

23 understood. Specifically, we do not know if there are any mechanisms by which

24 multicellularity can be maintained without a single cell bottleneck or other relatedness

25 enhancing mechanisms. Under low relatedness, cheaters can evolve that benefit from the

26 altruistic behaviour of others without themselves sacrificing. If these are obligate

27 cheaters, incapable of co-operating, their spread can lead to the demise of

28 multicellularity. One possibility, however, is that co-operators can evolve resistance to

29 cheaters. We tested this idea in a facultatively multicellular social amoeba, *Dictyostelium*

30 *discoideum.* This amoeba usually exists as a single cell but, when stressed, thousands of

31 cells aggregate to form a multicellular organism in which some of the cells sacrifice for

32 the good of others. We used lineages that had undergone experimental evolution at very

33 low relatedness, during which time obligate cheaters evolved. Unlike earlier experiments,

34 which found resistance to cheaters that were prevented from evolving, we competed

35 cheaters and non-cheaters that evolved together, and cheaters with their ancestors. We

36 found that non-cheaters can evolve resistance to cheating before cheating sweeps through

37 the population and multicellularity is lost. Our results provide insight into cheater-resister

38 co-evolutionary dynamics, in turn providing experimental evidence for the maintenance

39 of at least a simple form of multicellularity by means other than high relatedness.

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42 **Keywords: major transition, multicellularity, altruism, cooperation, cheaters,**

43 **experimental evolution.**

44   **Introduction**

45

46   *Multicellularity*

47   Perhaps the most interesting moments in the history of life are the great

48   transformations in the unit of individuality, when what were previously self-sufficient,

49   functioning individuals, become integrated into a collective, no longer capable of

50   replicating independently (Maynard-Smith and Szathmáry 1995). They are interesting in

51   large part because of the questions they raise about conflict. In order for a higher level of

52   biological organization to form, conflict must be controlled at lower levels. Consider, for

53   example, the origin of multicellularity, one of the six widely recognized major transitions

54   (Bourke 2011). Multicellularity requires anywhere from a few to many millions of cells

55   to sacrifice for the good of only a minority. Why do the cells in our hands, hearts, and

56   brains sacrifice their own reproduction so that our gametes can be passed on?

57   Inclusive fitness provides one answer (Hamilton 1964a;b). With each generation

58   the organism passes through a single celled bottleneck (i.e. the zygote), meaning that all

59   of the cells within the organism are clonally related. Their relatedness is one ($r = 1$), so

60   the genetic basis for conflict is effectively eliminated. In addition, any cheater mutation

61   that gets a cell into the germ-line will be limited to one round of cheating, because in the

62   following generation it will be found in a multicellular organism consisting entirely of its

63   clones (Queller 2000). Thus, inclusive fitness explains how multicellularity can be

64   evolutionarily stable and may also explain the prevalence of single-cell bottlenecks.

65   However, questions remain. First, there are alternative explanations for the

66   prevalence of single-cell bottlenecks; they might serve to purge deleterious mutations

67   (Grosberg and Strathmann 1998) or they might be necessary for complex development

68   (Wolpert and Száthmary 2002). The existence of single-cell bottlenecks therefore cannot

69   be taken as strong evidence for the importance of conflict reduction. Second, although

70   most examples of multicellularity have their origin in clonality, there are a few

71   exceptions, like the social amoeba, *Dictyostelium discoideum*. If multicellularity did not

72   originate in clonality, what level of relatedness would be required amongst the cells for

73   multicellularity to be stable? The examples provided by social insects show that

74   extremely cooperative entities (colonies) can be stable without clonal relatedness, in part

75   through coercion or policing (Ratnieks and Wenseleers 2007), a co-evolutionary response

76   of other parties to the evolution of cheating. Are there other forces like this at play that

77   could promote and maintain multicellularity? For example, could non-cheaters evolve

78   resistance to cheating? Buss (1987) argued that many aspects of multicellular

79   development evolved from such an interplay between cellular cheaters and resisters.

80   Some of these scenarios are implausible under high relatedness (Queller 2000), though

81   others, such as control of cell division rates, might not be (Michod 1997). But if

82   relatedness were low in the evolution of multicellularity, such co-evolutionary responses

83   to cheaters might be required for multicellularity to be maintained. Unfortunately, these

84   questions are difficult to explore experimentally. Most multicellular organisms obligately

85   pass through a single-cell bottleneck, such that their intra-organismal relatedness cannot

86   be experimentally manipulated. Since *D. discoideum* becomes multicellular by

87   aggregation, it is a great system in which to explore what is mostly the path not taken.

88

89   *Dictyostelium discoideum*

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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).

113

114   *Cheating of Multicellularity in Dictyostelium*

115   Kuzdzal-Fick *et al.* (2011), starting with a single clone, artificially maintained low

116   relatedness in *D. discoideum* for 31 rounds of vegetative growth, starvation and spore

117   formation. Low relatedness, maintained by starting each new generation with a random

118   mixture of 10^6 spores, allowed cheaters that appear by mutation to be favoured by

119   selection. These cheaters cheat by increasing their representation in the sorus, while

120   contributing little or nothing to the stalk.

121   Further, some of these evolved cheaters were obligate cheaters, which cannot

122   produce fruiting bodies on their own. Obligate cheaters, unlike facultative cheaters, do

123   not modulate their cheating based on their partners (Travisano and Velicer 2004; Ghoul

124   *et al.* 2014). Here, cheating entails not sacrificing to form the stalk. In mixtures this

125   works because the other, non-cheating clone forms the stalk. But when alone, an obligate

126   refusal to form stalk means that no spores form either (Ennis *et al*. 2000; Gilbert *et al.*

127   2007), so the organism has no fitness. The importance of this distinction is that co-

128   operation can persist in the presence of facultative cheaters, but obligate non-fruiting, if it

129   sweeps through the population, eliminates co-operation. Obligate non-fruiters also have

130   an added experimental value, because the cheaters can be readily identified when plated

131   out clonally, as they fail to fruit, simply forming a small group of cells.

132   Even though mutation rates to obligate non-fruiting cheaters are known to be low

133   (Hall et al. 2013), cheaters readily rise to high frequencies when intra-organismal low

134   relatedness provides them with fruiting victims to exploit (Kuzdzal-Fick *et al.* 2011). At

135   high relatedness, such as occurs in natural fruiting bodies, these mutants do poorly

136　(Gilbert *et al.* 2007). This raises a question about the evolution of multicellularity. If

137　relatedness were not high, would anything prevent cheating from sweeping through the

138　population? Several mechanism could be involved (Strassmann and Queller 2011), one of

139　which is that co-operators could evolve resistance to cheating. Khare *et al.* (2009)

140　demonstrated that resistance to cheating can be selected for when the cheaters are held

141　constant (not evolving). They presented *D. discoideum* populations with a cheater for

142　four cycles of selection. They showed that the repeated presence of the cheater selected

143　for resistance to cheating. This experiment suggests that it may be possible for co-

144　operators to evolve resistance to cheating. Hollis (2012) tested co-evolving populations of

145　*Dictyostelium* cheaters and non-cheaters, and populations of evolving non-cheaters

146　against non-evolving cheaters, and only found evidence for the evolution of resistance

147　when the cheaters were not allowed to evolve. Therefore, it has yet to be demonstrated

148　that a co-evolutionary response to cheating can evolve before cheating sweeps through

149　population and multicellularity is lost. Ideally we would like to know if resistance

150　evolves in real populations, with the cheaters and non-cheaters co-evolving in real time.

151　Our experiments explore this question.

152

153　*Resistance experiment*

154　　　　Our experiments test whether *D. discoideum* can evolve resistance to cheating

155　while cheaters are evolving, before the obligate cheating phenotype sweeps through the

156　population. We used lineages from the Kuzdzal-Fick (2011) experiment, which

157　underwent experimental evolution at low relatedness and evolved cheating. Non-fruiting

158　clones – potential obligate cheaters – increased in the experiment and three of four tested

159   against the (fruiting) ancestor were confirmed to be cheaters (Kuzdzal-Fick (2011). We

160   first confirmed that this ability to cheat the ancestor held for much larger numbers of non-

161   fruiting clones. Then, to test whether resistance had also evolved, we tested the non-

162   fruiters against fruiting clones isolated from their own selection lines. If resistance has

163   not evolved, we would expect to find the same proportion of non-fruiters in the sori of

164   both mixtures (evolved non-fruiters with ancestors and evolved non-fruiters with evolved

165   fruiters). If the evolved fruiters have evolved resistance to cheating we would expect

166   them to be better than the ancestors at keeping the non-fruiter out of the sorus.

167   Our results showed that resistance to cheating did evolve. Because we worked

168   with clones from populations that evolved from a single clone, this means that resistance

169   evolved after the obligate cheaters emerged but before they swept through the population.

170   This has implications for both our understanding of the evolutionary dynamics of

171   cheating and resistance (e.g. co-operators can evolve resistance to cheating in real time),

172   and our understanding of the evolution of multicellularity (e.g. there are mechanisms

173   other than self-limitation that can stabilise simple multicellularity).

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175

176   **Materials and Methods**

177   *Amoebas*

178   We used the ancestor and 4 experimental lines from the Kuzdzal-Fick *et al.* 2011

179   study. The experimental lines had gone through thirty-one rounds of fruiting, with each

180   round initiated by spreading a million cells across a new plate, so any new cheater

181   mutation would be well-mixed among victims (Kuzdzal-Fick *et al.* (2011)). All four of

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182 these experimental lines had a reported frequency of non-fruiting mutants of 50% or

183 more, which we verified with an initial plating of all the lines.

184   All lines had been frozen as spores in KK2 Buffer (per liter: 2.25 g KH_2HPO_4,

185 0.67 g K_2HPO_4) with 25% glycerol at -80 degrees Celsius, as described in the Supporting

186 Online Material for Kuzdzal-Fick *et al* (2011). For all frozen samples, we thawed them

187 gently (at room temperature), diluted quickly, counted spores using a haemocytometer,

188 and then plated 50 spores each onto 10 mL SM/5 plates with 200 µL of *Klebsiella*

189 *pneumoniae* in KK2 (OD600 1.5). Plated spores or plated amoebas took between 76-80

190 hours to form fruiting bodies. After fruiting, we allowed one week before collecting

191 fruiting bodies. Details of culturing can be found in Kuzdzal-Fick *et al.* (2011).

192

193 *Population Experiment*

194   The purpose of the population experiment was to determine whether or not there

195 was resistance to cheating in the evolved populations. We achieved this by competing

196 populations of putatively cheating evolved non-fruiters against both the ancestor (which

197 is a fruiter) and populations of evolved fruiters from the same line. Here population is

198 defined as a mixture of 25 separate clonal plaques of each type. If non-fruiters do cheat

199 the ancestor as expected, resistance to cheating will be established if they cheat the

200 evolved fruiters less or not at all. If the non-fruiter cheats both the ancestor and the

201 evolved fruiter equally, we would expect to see the same number of non-fruiters in the

202 sori for both mixtures. This experiment is illustrated diagrammatically in Figure 1.

203   In this experiment, for each line (Kuzdzal-Fick lines: 12, 16, 21, and 24), we

204 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.

228   Next we pooled spores from 4 fruiting bodies from each plate (evolved non-fruiter

229   with ancestor and evolved non-fruiter with evolved fruiter), and plated 1000 spores from

230   each plate onto 20 SM/5 plates with *K. pneumoniae* (20 plates per experiment mixture, 2

231   mixtures per line, 4 lines, 160 plates). We diluted the spores across so many plates

232   because we wanted to see individual plaques as fruiters or non-fruiters (our measure of

233   non-cheaters and cheaters). We allowed 76-80 hours for these to fruit, and then scored

234   each colony as either a fruiter or a non-fruiter. We counted the total number of fruiters

235   and non-fruiters for each experimental mixture, and used this to determine the proportion

236   of non-fruiters. This served as our measure of the degree of cheating, because it measured

237   how many of the non-fruiters ended up as spore rather than stalk cells.

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241   *Individual Experiment*

242   The purpose of the individual experiment was to confirm the results of the

243   population experiment at the individual level by looking for resistance to cheating in

244   individual evolved fruiter clones (rather than evolved fruiter populations), and to look for

245   variation among individual evolved fruiter clones. If individual evolved fruiter clones

246   have resistance, we would expect fewer non-fruiters to get into the sori in mixtures of

247   evolved non-fruiters and evolved fruiters than in mixtures of evolved non-fruiters and

248   ancestors. However, if not all evolved fruiters have resistance, or if there are different

249   kinds of resistance in the population, we would expect some mixtures of non-fruiters and

250   fruiters to yield the same number of non-fruiters in the sori as in mixtures with ancestors.

251   The individual experiment is depicted diagrammatically in Figure 2.

252   In the individual experiment we used the three lines that most clearly appeared to

253   have evolved resistance in the population experiment: 12, 16, and 21. We plated these

254   lines and the ancestor from the freezer at low density (50 spores on each of 5 plates for

255   the three lines). We allowed 76-80 hours for the amoebas to fruit, and then picked two

256   fruiter colonies and two non-fruiter colonies for each line. We cultured each clone

257   separately in HL5 + PSV as described previously (thus, four cultures for each line: non-

258   fruiter 1, non-fruiter 2, fruiter 1, and fruiter 2). We cultured ancestors in the same fashion.

259   We cultured the clones for three days. On the second day of culture, we split the

260   10 mL of culture into two plates. After three days of culture, we washed the cells of

261   antibiotic by centrifuging the cultures in 15 mL centrifuge tubes and adding fresh HL5

262   with no antibiotic three times. We then diluted the cells to $1x10^7$ cells mL^{-1}. We plated six

263   experimental mixtures from each line, testing each of the evolved non-fruiters against

264   both the evolved fruiters from its own line and the ancestor: Evolved Non-Fruiter 1 +

265   Evolved Fruiter 1, Evolved Non-Fruiter 1 + Evolved Fruiter 2, Evolved Non-Fruiter 2 +

266   Evolved Fruiter 1, Evolved Non-Fruiter 2 + Evolved Fruiter 2, Evolved Non-Fruiter 1 +

267   Ancestor, and Evolved Non-Fruiter 2 + Ancestor. The mixtures were a 75:25 ratio, with

268   the non-fruiter always making up 75% of the mixture. We also plated each clone on its

269   own as a control to ensure that each clone was still healthy after being in liquid culture

270   (and that there was no fruiter/non-fruiter contamination). We allowed ten days for the

271   fruiting bodies to reach the stage of having harvestable sori.

272   We then harvested all of the fruiting bodies for each mixture using a loop. We

273   diluted the spores from each mixture to 1000 spores in 4 mL of *K. pneumoniae* in KK2

274   (OD600 1.5). For each mixture, we plated 200 µL of solution onto each of 20 plates, with

275   the aim of having roughly 50 spores per plate. We allowed three days for the spores to

276   hatch, develop, and reach the fruiting stages so we could score them as fruiters or

277   nonfruiters.

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279

280   *Statistical Analyses*

281   We conducted all statistical analyses using R version 2.15.2 (released October

282   2012). We arcsine-square root transformed all proportion data (number of evolved non-

283   fruiter spores out of total spores in the sorus) to compensate for skew created by the 0 and

284   1 boundaries of proportional data. We analysed the data from the population experiment

285   with a paired t-test (95% confidence level). We analysed results from the individual

286   experiment using a Welch two sample t-test for samples of unequal sizes (95%

287   confidence level), and multiple two-tailed binomial tests (95% confidence level).

288   For the figures, we plotted relative sporulation efficiency against mixture.

289   Relative sporulation efficiency ratio is the ratio of cheater sporulation efficiency to

290   competitor (ancestor or evolved fruiter) sporulation efficiency. Sporulation efficiency is

291   calculated as fraction of spores in the sorus over fraction of cells in the initial mixture

292   (see Buttery *et al.* 2013).

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Results

Population Experiment

The population experiment tested for resistance to cheating in non-cheaters from

experimentally evolved populations that contained cheaters. We did this by testing

whether the evolved fruiters did better than the ancestral fruiters against the evolved

cheating non-fruiters.

302

In mixtures with the ancestor, the proportion of non-fruiter increased significantly

(two-tailed, one-sample t-test, t_3=6.75, p=0.007). The proportion of evolved non-fruiter

rose from 0.25 to a mean of 0.660 (95%CI: $0.463 < u < 0.832$), with their sporulation

efficiency about 7 times that of the ancestor (Figure 3). In contrast, the proportion of

evolved non-fruiter did not significantly change in mixtures with the evolved fruiters

(two-tailed, one-sample t-test, t_3=1.48, p=0.235). The mean proportion of non-fruiter rose

from 0.25 to a mean of 0.344 (95%CI: $0.155 < u < 0.563$). A paired t-test rejects the null

hypothesis that there is no difference in non-fruiter proportion between evolved fruiter

mixtures and ancestor mixtures (Figure 3, t_3=-8.29 p=0.004), and therefore supports the

evolution of resistance.

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314

Individual Experiment

The individual experiment tested for resistance to cheating in individual evolved

fruiter clones.

318   The proportion of evolved non-fruiters increased significantly in mixtures with

319   ancestors (two-tailed, one-sample t-test, t_5=11.04, p<0.001). The mean proportion of non-

320   fruiters increased from 0.75 to a mean of 0.916 (95%CI: $0.884 < u < 0.943$). The

321   proportion of evolved non-fruiters in mixtures with evolved fruiters did not significantly

322   change (two-tailed, one-sample t-test, t_{11}=-1.09, p=0.298). The mean proportion of non-

323   fruiters decreased slightly from 0.75 to 0.682 (Figure 4, 95% CI: $0.533 < u < 0.813$). A

324   Welch two sample t-test rejects the null hypothesis that there is no difference in mean

325   proportion between mixtures with ancestors and mixtures with evolved fruiters ($t_{12.9}$=-

326   4.22, p=0.001).

327   The proportion of non-fruiters increased significantly in six out of six ancestor

328   mixtures (Figure 5, $p < 0.05$, two-tailed binomial tests, 95% confidence level). However,

329   the tests of non-fruiters with evolved fruiters showed much more variation. In two of the

330   evolved fruiter mixtures (16 F1 + NF2, and 21 F1 +NF2), the proportion of non-fruiters

331   did not change significantly ($p = 0.564$ and $p = 0.143$, respectively, two-tailed binomial

332   tests, 95% confidence level). In four of the evolved fruiter mixtures, the proportion of

333   non-fruiters went up significantly, and in six of the fruiter mixtures, the proportion of

334   non-fruiters significantly decreased ($p < 0.05$, two-tailed binomial tests, 95% confidence

335   level).

336

337

338   **Discussion**

339   Our results add to a growing body of knowledge regarding the nature and

340   dynamics of cheating and resistance. Numerous studies have explored the nature of

341   cheating, both in *Dictyostelium* (e.g. Ennis *et al*. 2000; Strassmann *et al.* 2000; Buttery *et*

342   *al.* 2009; Buttery *et al.* 2013) and across a wide variety of other taxa. For example,

343   cleaner fish cheat by biting their host instead of cleaning (e.g. Bshary and Grutter 2002

344   for fish)*,* co-operatively scavenging *Pseudomonas* bacteria cheat by not producing costly

345   iron scavenging siderophore molecules (Griffin et al. 2004)*, Myxococcus* bacteria cheat

346   in cooperative spore formation (Velicer *et al*. 2000), and fork-tailed drongos mimic alarm

347   calls of pied babblers in order to gain access to food (Ridley *et al.* 2007; Flower 2011)

348   (see Ghoul *et al.* 2014 for a review of cheating). Our experiment follows up the

349   demonstration by Kuzdzal-Fick *et al.* (2011) that low relatedness leads to the evolution of

350   obligate cheating, and shows that this cheating is widespread, adding to the stack of

351   empirical evidence that relatedness is important for co-operation and prevents the spread

352   of cheaters. However, factors other than relatedness and kin selection can be important in

353   limiting cheating. This must be the case in between-species interactions where

354   relatedness cannot play a role. Thus, client fish will avoid cheating cleaner fish that bite

355   (Pinto *et al.* 2011) and legumes may shut off resources to nodules that fail to produce

356   nitrogen (Kiers *et al.* 2003). Even within species, cheating is sometimes controlled by

357   evolutionary responses among the cheated. For example in social insects, egg laying by

358   subordinates can be controlled either by dominant queens or through policing by other

359   workers (Queller and Strassmann 1998). Our experiments, which explored the co-

360   evolution of cheaters and resisters in evolving *D. discoideum* lineages, tested whether

361   cheater resistance could play this role in the evolution of multicellularity.

362   We first confirmed the result of Kuzdzal-Fick *et al.* (2011) that most non-fruiters

363   are obligate cheaters that cheat their ancestor. In the population experiment, the

364   proportion of non-fruiters in the sorus, on average, increased significantly in ancestor

365   mixtures (Figure 3). In the individual experiment, the proportion of non-fruiters increased

366   significantly in six out of six ancestor mixtures. The latter result brings the total for both

367   studies to nine out of ten.

368   Our main question is whether this evolution of cheating non-fruiters provoked a

369   co-evolutionary response among the fruiters. Both the population and individual

370   experiments show that fruiting clones that have evolved in the presence of non-fruiters

371   (evolved fruiters) are resistant to the evolved non-fruiters' cheating. In the population

372   experiment, there was no significant change in non-fruiter proportion in mixtures with the

373   evolved fruiter, and in both experiments, evolved fruiters did significantly better than the

374   ancestor when tested with non-fruiters.

375   A possible alternative explanation for this result is that the 31 extra rounds of

376   adaptation to the lab environment makes both evolved fruiters and non-fruiters better than

377   the ancestor. This seems very unlikely for two reasons. First, better adaptation to the

378   growth environment is not expected to enhance cheating; clones that produce more spores

379   on their own do not typically produce more spores per cell in mixtures (Buttery *et al.*

380   2009). Second, the clone used should already have been well adapted to the environment.

381   Kuzdzal-Fick *et al.* (2011) used a clone that is descended from strain NC4 collected in

382   the wild in 1933 (Raper 1984), so it had been in the laboratory environment for over 75

383   years where it has undergone extensive evolution (Bloomfield et al. 2008). The

384   experimental evolution took place in SM/5 medium with *Klebsiella aerogenes* on agar

385   plates (Kuzdzal-Fick *et al.* 2011), an environment that would have been commonly

386   encountered in those 75 years. Third, to further guard against possible issues of lab

387 adaptation, the Kuzdzal-Fick evolution experiment was initiated with a clone taken from

388 a line previously evolved in the exact same experimental evolution conditions for ten

389 rounds of fruiting and about 100 cell generations (Kuzdzal-Fick *et al.* 2011). The only

390 real novelty in the experimental evolution environment was the presence of cheaters due

391 to low relatedness. Fourth, if the lab adaptation hypothesis were true, it would imply the

392 strange result that, leaving aside the obligate cheating trait itself, the fruiters are

393 consistently evolving more rapidly to the lab than the cheaters are.

394   A potential test of lab adaptation by the evolved fruiters would involve competing

395 them against the ancestor, but this would require different and less comparable methods

396 because we could not assess frequencies via incidence of non-fruiting. Moreover, even if

397 it did show that the evolved non-fruiters did better against the ancestor, that would at

398 most add a dimension to our understanding of the selection (e.g. see Asfahl *et al.* 2015

399 for an example where the adaptation was non-social). It would not take away the

400 component we have demonstrated – that evolved fruiters resist cheating of the non-

401 fruiters and that this advantage would have been in play in the non-fruiter containing

402 environments where they evolved. Resistance cannot be said to be a side effect if it

403 played a demonstrable part in the selection.

404   Another possible explanation, though not really an alternative one, is that kin

405 recognition and segregation evolved during the course of the experimental evolution so

406 that when fruiters and non-fruiters are mixed, they segregate out and the non-fruiters do

407 poorly on their own. However, we did not see evidence of this. The lawns of fruiting

408 bodies from the mixture experiments were healthy and uniform, without defective

409 fruiting bodies. This is not surprising because, although *D. discoideum* does have some

410   degree of kin recognition (Ostrowski *et al.* 2008, Benabentos *et al.* 2009; Hirose *et al.*

411   2011), the resulting segregation is generally rather weak (Gilbert *et al.* 2012). It seems

412   unlikely that our clones would have evolved much stronger segregation in 31 rounds of

413   fruiting than natural clones have evolved over countless generations in the field.

414   However, the issue could bear further investigation, not as an alternative hypothesis, but

415   as one possible mechanism for the evolution of resistance to the non-fruiting cheaters.

416   In our experiment resistance evolved before obligate cheating could sweep

417   through the population, breaking down multicellularity. Prior experiments showing the

418   evolution of resistance either could not test this because they artificially kept the cheaters

419   from increasing or evolving (Khare *et al.* 2009), or failed to find co-evolution of

420   resistance, perhaps due to experimental design (Hollis 2012). In the latter case, the lack

421   of findings could be due to having only 10 generations of co-evolution, perhaps because

422   the main interest in that experiment was selection for cheating rather than resistance.

423   Our result offers a potential mechanism by which facultative multicellularity

424   remains stable. The lineages used were kept under artificially maintained low-relatedness,

425   so the experiment demonstrates that in the evolution of facultative multicellularity, if

426   there were no high population-structure to keep relatedness high, there could be another

427   mechanism by which multicellularity is maintained: the evolution of cheater resistance.

428   This might explain why no lineages went extinct in the original experiment, although

429   many were showing lower spore production (Kuzdzal-Fick *et al.* 2011). We cannot

430   exclude the possibility that they would go extinct given enough time, but the evolution of

431   resisters should at least slow the process. And even if extinction would ultimately occur

432   with near-zero relatedness, a modest degree of relatedness together with resistance

433   evolution might be sufficient to prevent extinction.

434   The individual experiment also suggests that there may be variation in resistance

435   phenotypes in the population. The proportions of non-fruiters were much more variable

436   in evolved fruiter mixtures than ancestor mixtures. In two of the mixtures no cheating

437   occurred, in four of the mixtures the evolved fruiter was cheated, and in six of the

438   mixtures the evolved-fruiter appeared to cheat the cheater. This raises the possibility

439   that, although the frequency of resistance in the population may be similar across

440   lineages, not all individuals have evolved resistance and there may be different

441   phenotypes for resistance.

442   These results provoke interesting questions about the nature of resistance. The

443   population experiment supports previous work (Khare *et al.* 2009; Hollis 2012) that

444   showed that resisters can be noble, meaning they do not cheat the cheater, they only

445   prevent it from cheating (Khare *et al.* 2009; Hollis 2012). Though this was true in our

446   experiments on average, in a number of our individual tests, the evolved fruiter was not

447   only resistant, but also ignobly cheated the non-fruiting obligate cheater.

448   Some cheater genotypes have already been identified, but little is known of the

449   mechanism by which cheating works (Santorelli *et al. 2013*), and nothing is known about

450   resister genetics or mechanisms. One question that remains is how frequency affects the

451   dynamics of co-evolution. In our cheating tests, we tested only one mixture frequency

452   but previous work with both non-fruiting and fruiting cheaters suggests that who cheats

453   does not generally change with frequency (Gilbert et al. 2008; Buttery et al 2009).

454   Another open question is the amount of variation in the different cheater/resister

455   phenotypes and how they interact with each other.   The results from the individual

456   experiment suggest that this would be a valuable path to pursue.

457   These questions are relevant to the broader research programme on co-

458   evolutionary arms races. There has already been some evidence of cheater-resister

459   evolutionary arms races (Ghoul *et al.* 2014). Among many examples, there is an

460   evolutionary arms race between brood parasitic cuckoos and their hosts, in which

461   cuckoos are selected to cheat their hosts through egg mimicry and their hosts are selected

462   to detect the deception (Davies 2000; Spottiswoode and Stevens 2010; Langmore *et al.*

463   2011; Stoddard and Stevens 2011). Although co-evolution was originally conceived for

464   cases like this that concern interactions between species, the concept has long been

465   extended to within-species reciprocal interactions, such as between the sexes (Arnqvist

466   and Rowe 2002) or between nuclear and cytoplasmic genes (Werren 1987). In our

467   experiment, in only 31 rounds of experimental evolution, non-fruiting and fruiting types

468   evolved in response to each other. These results suggest that a co-evolutionary arms race

469   could occur among cell types in facultatively multicellular organisms. This would be

470   particularly likely for resisters that are "ignoble", and cheat the cheaters. Pursuing this

471   avenue of research will add to our knowledge of cheating, resistance, and evolutionary

472   arms races more broadly.

473   Cancer may provide another example of where cheater-resister evolution is

474   important in the context of multicellularity. Cancerous cells can be considered cheaters at

475   the intra-organismal level (Nunney 1999; Bourke 2011; Ghoul *et al.* 2014).

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

477   maintains co-operation at the organismal level, is a potentially valuable approach for

478   research on cancerous cheats.

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481   *Conclusion*

482   Our findings demonstrate that, in *Dictyostelium discoideum,* non-cheaters can

483   evolve resistance to cheaters when both are evolving together, and that they can do so

484   before obligate cheating sweeps through the population and multicellularity is lost. This

485   offers a mechanism by which, even if low relatedness conditions occurred in the

486   evolution of facultative multicellularity, at least a simple form of multicellularity could

487   be stabilised by the evolution of resistance to cheating.

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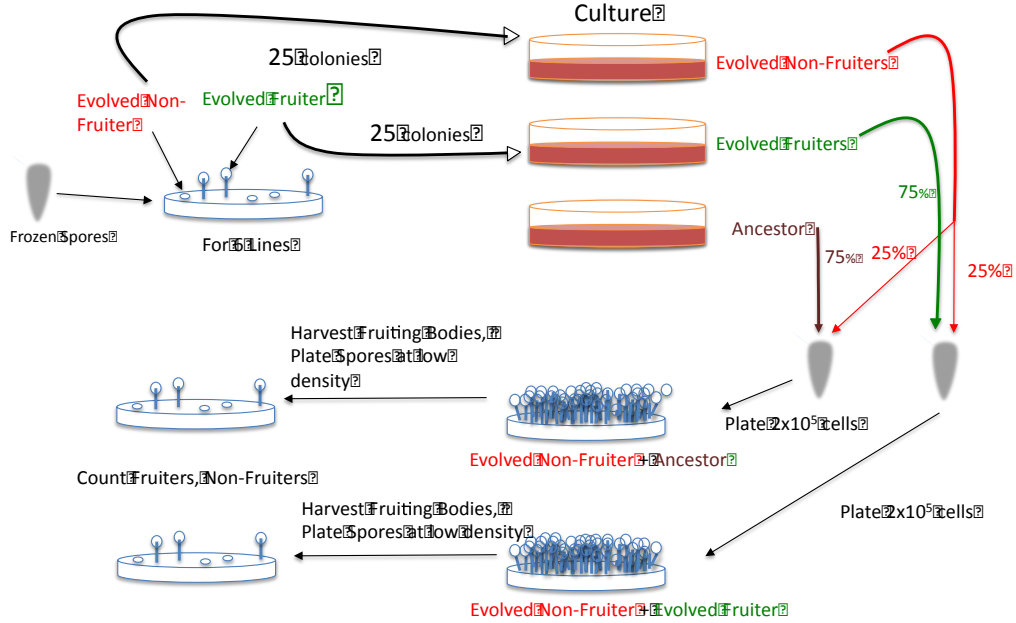
Figure 1. A diagrammatic representation of the population experiment. Blue plates are 10 mL SM/5 plates with 200 µL of *Klebsiella pneumoniae* in KK2 (OD600 1.5). Orange plates are 10mL tissue culture plates containing HL5 with PSV antibiotic. Micro-centrifuge tubes are 1.5 mL.
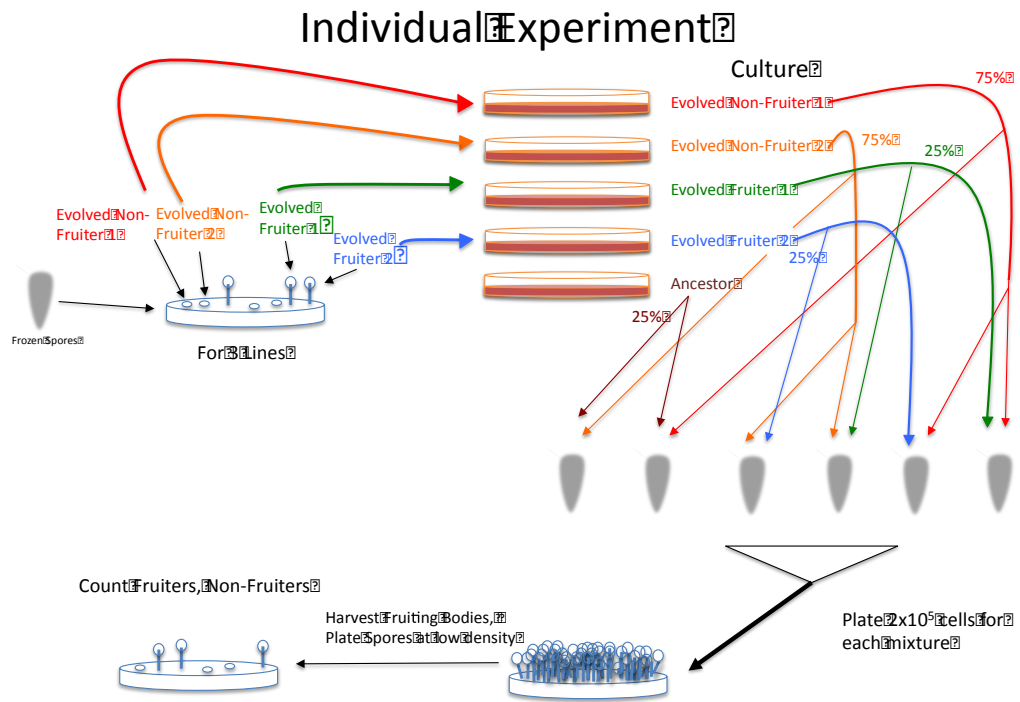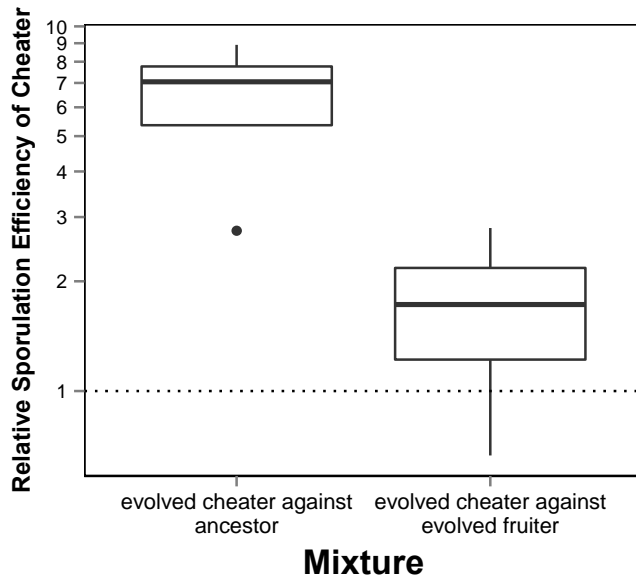
Figure 2. Diagrammatic representation of the individual experiment. Blue plates are 10 mL SM/5 plates with 200 µL of *Klebsiella pneumoniae* in KK2 (OD600 1.5). Orange plates are 10mL tissue culture plates containing HL5 with PSV antibiotic. Micro-centrifuge tubes are 1.5 mL.
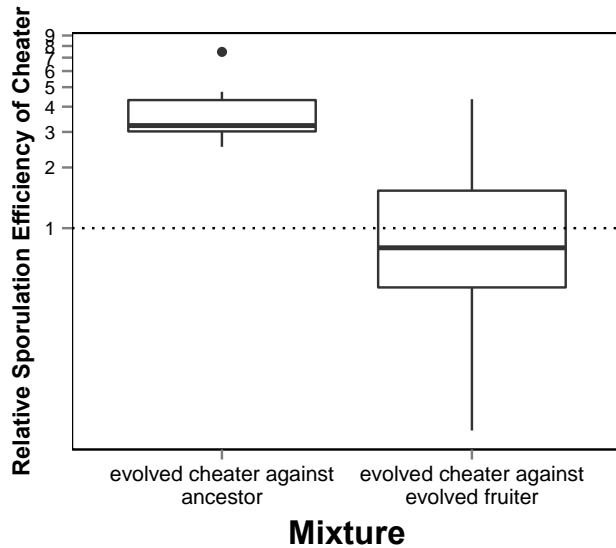
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653　Figure 3. Populations of evolved fruiters resist cheaters better than ancestors resist

654　cheaters. Plot of cheater sporulation efficiency ratio of non-fruiters in ancestor and

655　evolved fruiter mixtures (n=4). Outliers (1.5 times the inter-quartile range (IQR) greater

656　than the upper quartile or 1.5 times the IQR less than the lower quartile) are shown as

657　points. Y-axis is log scale. The cheater has a higher sporulation efficiency in ancestor

658　mixtures than in evolved fruiter mixtures (p=0.022, paired t-test). A relative sporulation

659　efficiency of 1 would be no cheating, and higher values suggest cheating has occurred

660　(dotted line at y=1 reference).

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Figure 4. Individual clones of evolved fruiters resist cheaters better than ancestors resist

cheaters. Plot of cheater sporulation efficiency ratio of non-fruiters 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

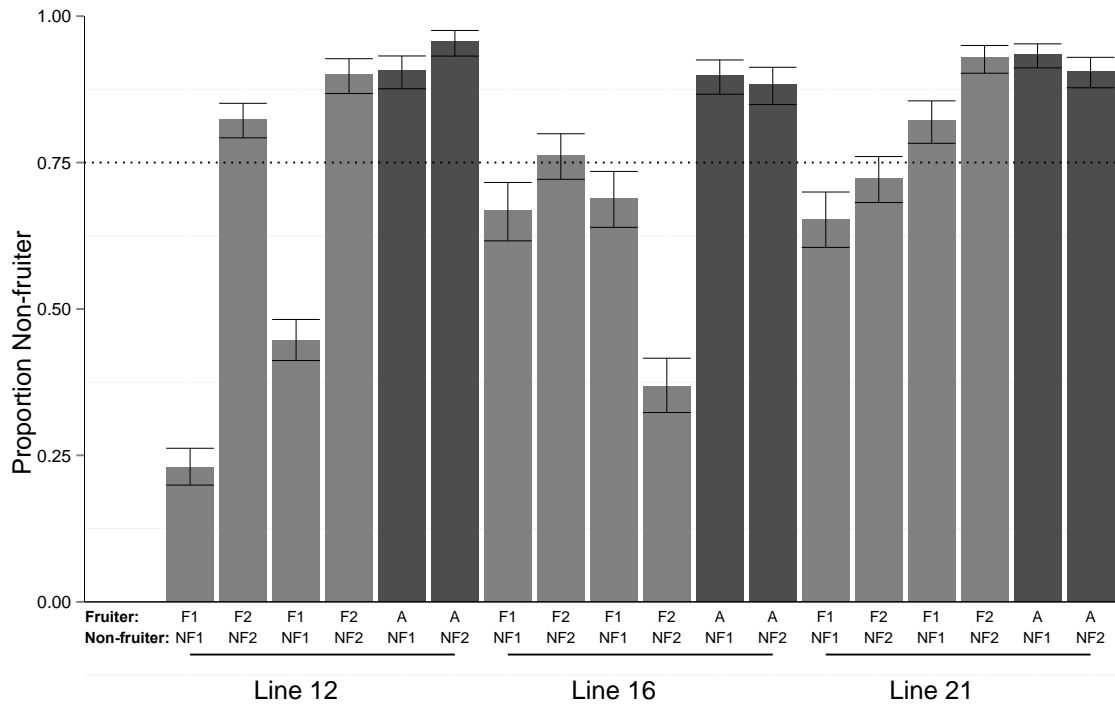1.5 times the IQR less than the lower quartile) are shown as points. Y-axis is log scale.

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).

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Figure 5. In the individual experiment, cheaters cheat the ancestor consistently but have

variable results with the evolved fruiters. Proportion non-fruiter in the sori resulting from

mixtures of the evolved non-fruiter with either the evolved fruiters 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.