Costs of being a diet generalist in the super-generalist protist predator *Dictyostelium discoideum*

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Author Contributions: P.M.S., J.E.S., and D.C.Q. designed research. P.M.S., D.A.B., and R.I.M. performed the experiments. P.M.S., J.E.S., and D.C.Q. analyzed data. P.M.S., J.E.S., and D.C.Q. wrote the paper.

Competing Interest Statement: The authors declare no competing interest.

Classification: Major - Biological Sciences Minor- Ecology

Keywords: Diet breadth, Generalist, Predation, Protists, Switching costs

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Abstract

Organisms can be classified as diet specialists or generalists based on the number of prey types they eat. Since different prey species have different constitutions and defenses, eating a wide variety of prey presents challenges. We explore three potential costs of generalism in a super-generalist amoeba predator, *Dictyostelium discoideum*, feeding on naturally co-occuring bacterial prey. First, we found that amoebas experience sub-optimal growth on many of their prey bacteria. Second, amoebas also exhibit a reduction in growth rate when they switch from one species of prey bacteria to another, something we call switching costs. These switching costs disappear within a day, indicating adjustment to new prey bacteria. Third, amoebas usually divide more slowly on mixtures of bacteria compared to their average on single bacteria, something we call concurrent costs. Our results support that idea that, although amoebas can consume a huge variety of prey, they must use partially different methods and thus must pay costs to handle multiple prey, either sequentially or simultaneously.

Significance Statement

Perhaps the most fundamental conflict in nature involves consumption of one organism by another. Diet generalists benefit from the advantage of eating many prey species but deal with many prey defences. We explore costs associated with a broad diet in a protist microbial predator, *Dictyostelium discoideum*. These predators of bacteria show a delay in growth when switched from one prey to another, supporting the hypothesis that they must deploy different strategies. They also experience costs when grown on many bacteria at once, suggesting that the alternative strategies for different prey are partly incompatible with each other. Our findings shed light on the nature of diet generalism and highlight the complexity of predation in the microbial world, where most organisms reside.

Main Text

Introduction

Organisms vary widely in their diet breadth. Some are diet specialists that eat one or few prey types, such as koalas that feed only on eucalyptus leaves (1) or snail kites that exclusively hunt apple snails (2). Some organisms are diet generalists that eat many different prey, such as coyotes that feed on many small mammals (3) or spiders that feed on many species of arthropods (4, 5). Diet generalists benefit from the ability to exploit diverse prey species, especially when prey species are scarce and fluctuate in their availability (6). But they must deal with prey that often differ in nutritional quality and defensive mechanisms. Efficiently handling these diverse prey is potentially hindered by trade-offs, which could select for diet specialization (7, 8). Have generalist predators overcome costs associated with consuming multiple prey species or do they experience costly trade-offs?

Many macroscopic predators consume a formidable number of prey species, but few can match the diversity of prey consumed by some microbial protist predators (9, 10). Predation by protists is a major factor accounting for bacterial mortality in the environment and plays an important role in nutrient cycling (9, 11, 12). This can determine the composition and properties of bacterial communities (13). Predation by protists is also an important selective pressure for bacterial defenses such as biofilm formation, antibiotic production, and secretion systems (14, 15).

Dictyostelium discoideum is one such super-generalist protist predator. *D. discoideum* is a social amoeba that lives in forest soils. It is a unicellular amoeba when bacterial prey are abundant and transitions to a non-feeding multicellular dispersal stage upon starvation. *D. discoideum* has been the subject of extensive research for this fascinating multicellular stage

65 (16, 17). Its feeding behavior has been less extensively studied, but it appears to qualify as a
66 super-generalist predator. It can eat the majority of bacteria it is presented with (18–20). For
67 example one study tested 159 bacterial strains found in close association with fruiting bodies of
68 *D. discoideum* from forest soil habitats and found that the amoebas were able to consume 77%
69 of them (20). Given the sheer diversity of bacteria in the soil – estimated to be as high as a
70 million species in 30 g of forest soil (21) – the number of edible species must be very large.

71 Specializing on one or few species of bacteria is probably not a viable option for *D.*
72 *discoideum*. This is because soil bacterial communities are spatially and temporally variable,
73 such that no one bacterium may be consistently sufficiently abundant (22). Indeed, even as
74 generalists starvation must occur often enough for it to evolve as the trigger for their
75 multicellular dispersal stage. Additionally, amoebas can travel considerable distances during
76 their social cycle through slug migration and especially through the dispersal of spores by animal
77 vectors (23). This can introduce them to new soil environments with different bacterial
78 communities. Thus, environmental heterogeneity likely plays a large role in the maintenance of
79 a generalist strategy in *D. discoideum*.

80 But handling a highly diverse array of prey bacteria is likely to be challenging for *D.*
81 *discoideum*. These prey bacteria are not identical and can range across four highly divergent
82 bacterial phyla: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (19). These shared
83 a common ancestor ~3000 million years ago, far older than the divergence time of diverse insect
84 prey (~400 mya) that generalist vertebrates feed on (24). Bacteria also possess highly varied
85 defensive mechanisms against microbial predators (14, 15). They can produce many kinds of
86 secondary metabolite toxins to repel, disable, or kill their enemies. They can use secretion
87 systems and effectors that can kill or allow for intracellular survival within protists. Some

88 bacteria can swim away at high speeds to escape predators. Some can group together to form
89 biofilms to prevent ingestion by predators.

90 We investigated three potential costs of *D. discoideum*'s broad diet. Each could reduce
91 the obvious benefit of being able to eat many species of prey bacteria. First, it is likely that not
92 all bacteria are equally profitable. The bacteria can differ in their nutritional quality and/or in
93 their susceptibility to the amoeba's hunting and processing techniques. Therefore, the first step
94 to understanding generalism of *D. discoideum* is to assess its performance on diverse prey.

95 Second, although generalist amoebas benefit from the ability to switch to a new prey
96 species when prey abundance changes, prey switching could be costly. If amoebas use different
97 methods to hunt and consume different prey, then changing between these methods can result
98 in temporary costs. These methods may be related to prey detection, foraging, detoxification,
99 and processing (25, 26) and may involve changes from the transcriptional level on up. Amoebas
100 do express partially different transcriptomes on different bacteria (27, 28). We refer to this
101 reduction in foraging efficiency as a switching cost.

102 Finally, in spatially fine-grained soil habitats, amoebas that consume multiple prey at
103 once could experience costs if specific techniques work best for different prey. It may be
104 impossible to effectively deploy multiple optimal strategies at the same time. We refer to the
105 reduction in foraging efficiency in the presence of multiple prey species as concurrent costs.
106 Alternatively, amoebas could benefit in this scenario due to improved nutritional balance. Some
107 studies have found an increase in protist growth rates in the presence of multiple prey species,

where mixed prey communities represent a balanced diet and can better fulfill the nutritional requirements of the predator (29, 30).

Results

Growth rate of *D. discoideum* amoebas varies on different species of bacteria.

We measured the growth rate of *D. discoideum* on 22 species of soil bacteria and the commonly used food bacterium *K. pneumoniae*. *D. discoideum* amoebas showed wide variation in their doubling times on different soil bacteria (Test bacterium: $F_{22,45} = 23.88$, p-value $< 2.2\text{x}10^{-16}$) (Fig 1). They doubled within four hours on some of the most edible soil bacteria and took longer than nine hours on some of the least edible soil bacteria.

D. discoideum amoebas experience switching costs on all tested bacteria pairs.

We conducted switching cost experiments to investigate how *D. discoideum* amoebas proliferate on a given bacterium when previously conditioned to a different bacterium (Fig 2). As expected given previously observed variation in growth rate of *D. discoideum* on different species of bacteria (Fig 1), the total number of amoebas at 3 hours for both switches and controls depended on the test bacterium (Test bacterium: $F_{5,106} = 28.39$, p-value $< 2\text{x}10^{-16}$). But independent of this effect, there were also switching costs; we found that the total number of amoebas at 3 hours from transfer significantly differed between the control and switches (Treatment: $F_{1,106} = 27.621$, p-value $= 7.69 \text{ x } 10^{-7}$). The switched amoebas performed poorly on a given bacterium compared to controls that did not experience a prey switch (Fig 3). There was no evidence that bacteria differed in switching costs as we found no significant interaction

between bacterial species and Treatment (Treatment x Test bacterium: $F_{1,106}$= 0.838, p-value = 0.526). There was no evidence that conditioning time of amoebas (2 days or 5 days) affected switching costs (Treatment x Conditioning length: $F_{1,106}$ = 0.543, p-value = 0.463). This is inconsistent with switching costs being due to evolutionary changes during conditioning since evolutionary changes should be greater over 5 days than 2.

D. discoideum do not experience switching costs if they undergo spore formation before the prey switch

If switching costs are because of an evolved response in amoebas, then these genetic changes should be passed on through the spores. But if the costs are physiological, then undergoing the social cycle will likely erase the effects of food conditioning and therefore eliminate switching costs. We therefore tested if *D. discoideum* still show switching costs when they undergo the social cycle and spore formation before changing between prey bacteria. The switched and control spores resulted in a similar number of amoebas (Fig 4, Treatment: $F_{1,48}$ = 0.263, p-value = 0.61). Thus, we find no support for the hypothesis that switching costs are due to an evolved response. But as expected, the total number of amoebas depends on the test bacterium the spores were grown on (Test bacterium: $F_{5,48}$ = 186.4, p-value < $2x10^{-16}$).

Switching costs are temporary

If switching costs are not due to an evolved response, as supported by our previous results, then the switched amoebas should eventually recover their growth rates to the level of the controls

151 that did not switch to new food bacteria. We conducted a time course experiment to test this
152 for one pair of bacteria (*A. tumefaciens*, *P. vranovensis*) and compared early (zero to six hours)
153 and late (24 to 27 hours) growth rates. The switched treatments had significantly lower early
154 growth rates compared to controls (Fig 5A, Treatment: $F_{1,17}$ = 9.458, p – value =0.007), as
155 expected from our earlier experiments. But there was no difference in late growth rate of
156 switches and controls at the later time points (Fig 5B, Treatment: $F_{1,17}$ = 0.111, p-value = 0.73).
157 Thus, though switched amoebas proliferate slower than controls during early growth, they
158 eventually catch up and perform similarly to controls, consistent with physiological adjustment
159 to the new prey.

160

161 ***D. discoideum* amoebas experience concurrent costs in some multi-prey communities**

162 We tested if *D. discoideum* experiences costs when grown in multi-species prey communities
163 compared to expectations from their growth in single-species communities. We refer to these
164 costs as concurrent costs. Consistent with concurrent costs, we observed significantly fewer
165 amoebas than expected in multi-species communities (Fig 6, Treatment: $F_{1,59}$=11.83, p-value =
166 0.001). We also found that total number of amoebas depended on which bacterial community it
167 was plated with (Bacterial community: $F_{5,59}$ = 8.74, p-value = 3.01x 10^{-6}). The interaction
168 between treatment and bacterial community was also significant, indicating that the identity of
169 the bacterial community influenced the degree of concurrent costs (Treatment x Bacterial
170 Community: $F_{5,59}$ = 2.557 p-value = 0.036). The general trend is in the expected direction in five
171 out of the six bacterial communities with one community trending in the opposite direction.

172

Discussion

Predators and prey engage in the most ancient of battles. Though largely unseen and understudied, the microbial stage for this conflict may be the largest of all. Here we have examined a key feature of this struggle: costs and benefits of specializing on few prey as compared to embracing a catholic diet. The eukaryote *D. discoideum* lives in heterogenous soil environments where it encounters many species of prey bacteria. In such a variable environment with shifting prey abundance, the benefit of being a diet generalist is obvious. The cost to generalism is the struggle to defeat a wide variety of defense mechanism as prey work to defend themselves. We found that amoebas experience varying growth rates when presented with the same quantity of different prey bacteria (Fig 2). We opted to equalize bacterial quantity across different prey by using optical density. Bacteria can differ in how they influence optical density, especially through their varying cell sizes (31). But optical density is a much more robust measure of dry weight (31) which is probably a more relevant measure of nutritional potential of bacterial prey. Previous studies of *E. coli* show that different strains vary substantially in nutritional quality to *D. discoideum*, and this result holds whether the bacteria are measured by OD (32) or by cell number (33). Thus, we have evidence that bacterial prey differ in nutritional quality for the amoebas. This is perhaps not surprising, especially given that there are also some bacteria that *D. discoideum* cannot grow on at all (20).

Amoebas experience switching costs when moved from one prey bacterium to another, even after controlling for variation in bacterial edibility (Fig 3). We did three experiments to test if these costs were physiological in nature or due to an evolved response. First, we found no evidence that the conditioning time (duration spent by amoebas on a given bacterium before the prey switch) affected the magnitude switching costs. If the costs were due to evolution

196 during the conditioning period, they should become stronger with longer conditioning time.
197 Second, we found no evidence that amoebas experience switching costs if they undergo spore
198 formation before prey switch. If this was due to an evolved response, then these effects would
199 be genetically passed on through the spores. Third, we found that switching costs occur during
200 early growth but disappear within a handful of generations, faster than they would change by
201 selection. Taken together these experiments show that these costs are physiological in nature,
202 likely to be associated with the need to alter gene expression on different prey bacteria, and not
203 due to evolution.

204 We also found that *D. discoideum* amoebas experience concurrent costs, where they
205 proliferate less than expected in communities with multiple species of prey bacteria. It is
206 interesting that we observe these costs despite two factors that may obscure them. First, some
207 mixed bacterial prey communities may provide a more balanced diet that could increase growth
208 rate of *D. discoideum*. Some studies on other protists that have looked at growth-rate in multi-
209 prey communities have found an increase in growth rates (29, 30). Second, the amoebas may
210 also choose to eat the most profitable bacteria first. All but late measurements would reflect the
211 growth rate of the best bacterium and any concurrent costs might therefore be obscured.
212 Amoebas do show preferential attraction towards Gram-negative bacteria compared to Gram-
213 positive bacteria (34). Thus, it would be interesting to test if *D. discoideum* amoebas can avoid
214 some concurrent costs by preferentially eating the most profitable prey bacteria first, but if they
215 do, our data show that it is apparently not enough to fully overcome such costs.

216 Both switching and concurrent costs are consistent with the idea that *D. discoideum*
217 amoebas use partially different methods to hunt and process different prey. Previous studies
218 have shown that *D. discoideum* amoebas transcribe partially distinct sets of genes on Gram-

219 positive (*B. subtilis*, *S. aureus*) and Gram-negative bacteria (*K. pneumoniae*, *P. aeruginosa*) (27).
220 The transcriptome of *D. discoideum* was also found to be highly species-specific when tested on
221 3 species of bacteria: *K. pneumoniae*, *B. subtlis*, *M. luteus* (28). These variable transcriptional
222 responses suggest that there are different mechanisms for hunting or processing these distantly
223 related bacteria. Another study found that mutations that affect the ability of *D. discoideum* to
224 grow on different bacteria were highly prey-specific (35).

225 Predation by amoebas is a complex process that can be divided into 4 broad steps:
226 search, encounter, attack, and digestion (14). Both switching and concurrent costs could arise in
227 any of these steps. Eukaryotic phagocytes use G-protein coupled receptors (GPCRs) to detect
228 and chase bacteria and use pattern recognition receptors to recognize and eliminate bacteria
229 (36, 37). *D. discoideum* is equipped with 61 GPCRs (38). Some GPCRs are involved in the
230 amoebas' social cycle, but at least one GPCR, the folate receptor fAR1, has been implicated in
231 both chasing and engulfing *Klebsiella* (39). Other GPCRs may play a role chasing and recognizing
232 different species of bacteria. *D. discoideum* is also equipped with 22 genes that may encode 4
233 different types of lysozymes (40). Lysozymes play an important role in attacking and digesting
234 bacteria. Knocking out some lysozyme genes generally reduces the ability of amoebas to feed on
235 all tested bacteria, while deletion of others reduces growth only on specific gram-negative
236 bacteria (41). Future research could involve identifying at what stage of predation these costs
237 are incurred.

238 Why can't amoebas evolve one predation technique that works optimally on all prey
239 bacteria to eliminate the costs of diet generalism? After all, amoebas are a few hundred times
240 larger than the average bacterium by mass (42). They should be able to overwhelm most
241 bacteria with their size advantage. But bacteria possess many mechanisms to resist their

242 eukaryotic predators (14, 15, 43, 44). Morphological adaptations such as formation of
243 microcolonies, biofilms, and filamentation can prevent attack or ingestion by predators. Bacteria
244 can modify their membrane properties by changing the lipopolysaccharides on the outer
245 membrane, secreting an S-layer and sporulating among others that can help them avoid
246 recognition, ingestion and digestion by predators. They can also produce a huge array of
247 secondary metabolites that can deter predation by protists (45, 46). Thus, each species of
248 bacterium may possess a unique combination of defenses that is unlikely to yield to a common
249 predation strategy. It would be interesting to test how many different predation strategies *D.*
250 *discoideum* can employ. *D. discoideum* may treat some groups of bacteria similarly and some as
251 different.

252 In this paper, we have demonstrated that *D. discoideum* experiences switching costs,
253 concurrent costs, and costs of feeding on sub-optimal prey. Many interesting questions remain
254 for future research. It remains to be seen how often amoebas experience these costs in nature.
255 This depends on the patchiness of bacteria on a microscale. One might expect that amoebas
256 have plenty of choices because soil habitats are extremely high in both bacterial diversity and
257 abundance. But on a microscale, soil is made up of small aggregated particles that are
258 connected via a network of air- and water-filled pores (47). The three-dimensional nature of
259 these particles increases soil surface area such that as little of 10^{-6} percent of soil surfaces may
260 contain bacteria (48). These particles generally contain bacterial patches of a limited number of
261 clonal cells and these patches can be separated by distances that are large on a microbial scale
262 (49). However, upper layers of soil can contain large bacterial patches comprised of different
263 morphotypes. Thus, it is possible that amoebas are likely to feed on sub-optimal prey bacteria
264 sometimes and may experience switching costs as they move between bacterial patches.

Most of what is known about predation comes from large organisms, but most organisms are microbes. The study of interactions between *D. discoideum* and its prey bacteria presents an exciting new frontier to investigate the mechanisms that underlie the costs and benefits associated with a generalist strategy. Microbial systems are particularly amenable to experimentation of all kinds in the lab. Questions on the strategies of predator and prey can be answered. Choice among bacterial prey, genetic bases of predation and resistance to it, and ecological foraging decisions can all be addressed. Our findings here contribute to this rich and exciting area of research.

Materials and Methods

Growth rate of *D. discoideum* on different species of bacteria isolated from forest soil environments

To investigate costs of eating sub-optimal prey, we first measured the growth rate of wild clones of *D. discoideum* on *Klebsiella pneumoniae,* the lab food bacterium, and on 22 species of bacteria found in close-association with *D. discoideum* isolated by Brock et al. 2018 from forest soil environments (Table 1). The bacterial species identification is based on closest partial 16S BLAST hit by Brock et al. 2018. In this paper, we refer to the bacteria using these closest 16s BLAST hits. We performed this experiment on 3 *D. discoideum* strains: QS1, QS6, and QS9. We plated 100,000 amoebas with 200µl of 10 OD_{600} bacterial suspension on starving agar plates. We estimated amoeba numbers at 20 hours and calculated doubling times as:

$$Doubling\ time\ (T_D)\ = ln(2)\ \times \frac{20}{ln(N_{t=20}) -\ ln(N_{t=0})}$$

286 This assay also helped us identify reasonably edible bacteria for other experiments.

287

288 **Switching cost assay in *D. discoideum* amoebas**

289 To test if switching costs occur when *D. discoideum* amoebas are switched from Bacterium A to
290 Bacterium B, we first conditioned separate populations of the amoebas to each species of
291 bacterium (Fig 2, details below for three separate experiments). The switched treatment for
292 these experiments used amoebas conditioned to Bacterium A and replated with Bacterium B.
293 We plated 100,000 amoebas conditioned to Bacterium A with 200μl 20 OD_{600} suspension of
294 Bacterium B on SM/20 plates and then measured amoeba numbers after 3 hours. The controls
295 for this experiment were identical except that amoebas conditioned to Bacterium B were
296 replated with Bacterium B. If we observe fewer amoebas in switched plates compared to control
297 plates, then *D. discoideum* amoebas experience switching costs. We measured the costs of prey
298 switching between 3 pairs of bacteria with 2 reciprocal switches for each pair. We replicated this
299 experiment with 5 *D. discoideum* strains: QS6, QS9, QS14, QS17, QS160.

300 We did three sets of experiments to rule out the possibility that switching costs are
301 instead due to an evolved response in the amoebas during the conditioning period to either
302 bacterium. Switched amoebas could experience poor growth on a Bacterium B because of trade-
303 offs associated with new evolutionary adaptation to the Bacterium A during the conditioning
304 period. Control amoebas may also experience better growth rates if they have evolved and
305 adapted to Bacterium B during the conditioning period. We ruled out this complication in three
306 ways.

307

308 **Effect of conditioning length of amoebas on switching costs**

309 First, we tested if the length of the conditioning period of amoebas on the bacterium affected
310 switching costs. If switching costs are due to either adaptation in switched amoebas that results
311 in evolutionary trade-offs, or to direct improvement in growth rate because of adaptation in
312 control amoebas, we expect stronger costs with longer conditioning (i.e. evolving) time. Thus,
313 we conditioned amoebas on a given bacterium for a) 2 days and b) 5 days before measuring
314 switching costs. In the 2 day treatment, we conditioned the amoebas for 40 hours by plating
315 200,000 spores of *D. discoideum* with 200ul of 1.5 OD_{600} bacterial suspension on SM/5 plates.
316 After ~40 hours, we washed off the plates with 10ml of ice cold KK2 to collect the amoebae-
317 bacteria suspension. Next, we centrifuged the suspension for 3 minutes at 300g at 10°C to spin
318 down the amoebas and discarded the supernatant containing the bacteria. We resuspended the
319 amoeba pellet in ice-cold KK2 and washed off two more times until all the bacteria were
320 discarded. Finally, we suspended the amoeba pellet in 500μl – 1000μl of ice-cold KK2. We
321 conditioned the amoebas for the 5-day experiment similarly, but collected amoebas from the
322 resulting vegetative front after 5 days with a sterile loop and resuspending in 600ul ice-cold KK2.
323 We performed washing centrifuging steps as described above to thoroughly wash the amoebas
324 off the conditioning bacterium. A conditioning time of 2 days translates to 10-14 amoeba
325 divisions and 5 days to 30-40 amoeba divisions on good prey. If switching costs are because of
326 an evolutionary response, then we expect costs to be stronger for the 5-day conditioning period
327 than the 2-day period.

328

329 **Switching costs assay with *D. discoideum* spores**

330 To further distinguish between a temporary switching cost and an evolved response, we tested
331 if allowing amoebas to go through the social cycle and subsequent sporulation before prey
332 bacteria switches erases switching costs (Fig 2). If switching costs are because of an evolved
333 response in amoebas, then these changes should still be evident after the social stage. But if the
334 costs are because of conditioning of the amoebas that results in temporary mismatch in
335 transcriptional tools on the new bacterium, then undergoing the social cycle should erase most
336 switching costs. This is because *D. discoideum* experiences a huge turn-over in gene expression
337 when transitioning from a unicellular lifestyle into the multicellular cycle (Parikh et al. 2010,
338 Rosengarten et al. 2015). The abundance of almost every transcribed mRNA changes at least 2-
339 fold throughout development starting from vegetative amoebas to multicellular fruiting bodies
340 (50). Therefore, any transcriptional conditioning of amoebas towards a bacterium should be
341 largely erased by development. The switched and control spores are at the same transcriptional
342 start line of dormancy.

343 We used the same design as the switching experiment for amoebas described above but
344 with sporulation at the end of the conditioning phase (~7 days). *D. discoideum* amoebas feed on
345 bacteria for the first 2-4 days of the conditioning phase, and then transition to the social cycle
346 once the bacteria are depleted. We then plated 100,000 spores from the resulting fruiting
347 bodies with bacterial suspensions on SM/20 (Fig 2). Since the spores require some time to
348 germinate into amoebas and start feeding on the bacteria, we counted the total number of
349 amoebas on these plates after 36 hours.

350

351 **Time course data of switching costs in *D. discoideum* amoebas**

352 As a third check on whether what we see is an evolved or a conditioned response, we conducted
353 a time series study on one pair of bacteria (*A. tumefaciens* and *P. vranovensis*) to check if the
354 growth rates of the switched treatments quickly catch up with control growth rates, which
355 would indicate a physiological lag rather than an evolutionary change. We tracked the number
356 of amoebas in switched and control plates after 6, 24 and 27 hours from plating. We calculated
357 early growth rate between 0h and 6h time points and calculated late growth rate between 24h
358 and 27h time points. We assumed exponential growth and used this formula to calculate growth
359 rate between time points t1 and t2, where N_t stands for number of amoebas at time t:

360 $$Growth\ rate\ (r)\ =\ \frac{ln(N_{t2})\ -\ ln\ (N_{t1})}{(t2\ -\ t1)}$$

361 If switching costs are not an evolved response, then switches and controls should not differ in
362 their late growth rates. We performed this on SM/5 plates with a starting number of 100,000
363 amoebas with 200µl of 1.5 OD_{600} suspension of the test bacterium on the *D. discoideum* clone
364 QS9. We replicated this experiment five times.

365

366 **Concurrent costs in *D. discoideum* amoebas**

367 Next, we determined whether *D. discoideum* amoebas experience concurrent costs of preying
368 on multiple bacteria. For this experiment, we chose 5 prey bacteria that were generally highly
369 edible from our growth rate assay of amoebas on 23 species of bacteria. We generated 5 single-
370 species communities, 6 three-species communities and 1 five-species community from the 5
371 prey bacteria (Table 3). We made OD10 suspensions of each bacterial species and mixed these

372 suspensions in equal proportions to make a given multi-species suspension. We used AX4
373 amoebas that were grown axenically in bacteria-free cultures to preclude any effect of prior
374 conditioning of amoebas to a given species of bacterium on the assay. We plated 100,000
375 amoebas on starving agar plates with 200μl of bacterial suspensions and measured amoeba
376 numbers in all the different treatments after 20 hours. We replicated this experiment 6 times.
377 To test if amoebas experience concurrent costs, we first calculated the expected number of
378 amoebas in multi-prey treatments with data from our single-prey treatments. For example, the
379 expected number of amoebas in multi-prey treatment containing *A. tumefaciens*, *S. maltophilia*
380 and *C. testosteroni* is the average of observed numbers of amoebas in single-prey treatment of
381 those bacteria. We then compared these expected numbers to our observed number of
382 amoebas in these multi-species treatments to infer concurrent costs.

383

384 **Statistics:**

385 We performed all statistical analysis in R (version 4.2.1) (52). To test if *D. discoideum* amoebas
386 experience varying doubling times on different species of bacteria, we used a one-way analysis
387 of variance with amoeba doubling times as the response variable and the Test bacterium (23
388 species of bacteria listed in Table 1) as the fixed factor (Table S1). We define Test bacterium as
389 the bacterium on which we measured amoeba growth rates. To test if *D. discoideum* amoebas
390 experience a switching cost on a given bacterium when previously grown on a different
391 bacterium we used a three-way analysis of variance (ANOVA) with log transformed amoeba
392 numbers after 3 hours of growth as the response variable and Treatment (Control/Switch), Test
393 bacterium (At, Pv, Fg, Sm, Sz, Ct), and Conditioning length (2days/5days) as fixed factors. We

also included interaction effects for Treatment x Test bacterium, Treatment x Conditioning length (Table S2). For the similar experiment that includes a spore stage after conditioning, we used a two-way analysis of variance (ANOVA) because there was no variation in conditioning length. Log transformed amoeba numbers after 36 hours was the response variable and Treatment and Test bacterium were the fixed factors. We included interaction effects for Treatment x Test bacterium (Table S3).

We performed the following statistical tests on time-course data collected on (At, Pv) switches. To test if there are switching costs during early proliferation we used a two-way analysis of variance (ANOVA) with early growth rate calculated between 0 and 6 hours as the response variable and Treatment (Control/Switch) and Test bacterium (At, Pv) as fixed factor (Table S4). We performed a similar two-way ANOVA with late growth rate calculated between 24 and 27 hours as the response variable to test if switching costs persist during late proliferation (Table S5).

To test if *D. discoideum* amoebas experience concurrent costs, we tested if amoebas performed worse than expected in multi-species prey communities. We used a three—way analysis of variance (ANOVA) with log transformed amoeba numbers at 20 hours as the response variable, Treatment (Expected numbers/Observed numbers) and Bacterial Community (6 Communities) and Experimenter (2 Experimenters) as fixed factors. We included an interaction effect between Treatment x Bacterial Community (Table S6).

Acknowledgments

This material is based upon work funded by the National Science Foundation under grants

IOS 16-56756, DEB 17-53743, and DEB 2237266. We thank Margaret Steele and Calum Stephenson for comments on the manuscript. We thank Trey Scott for feedback on statistical analysis. We would also like to thank the Queller-Strassmann lab for their helpful insights on experimental design.

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Figures and Tables

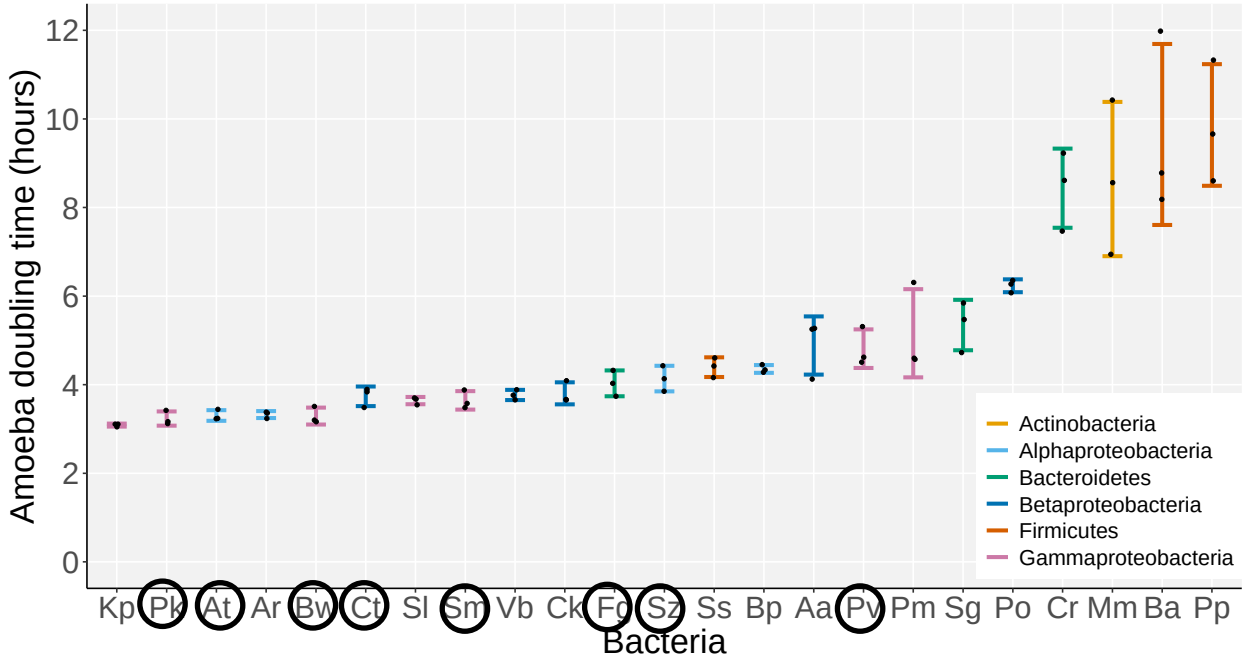


Figure 1. Doubling time of *D. discoideum* on *K. pneumoniae* and 22 species of soil bacteria closely associated with *D. discoideum*. Error bars are mean amoeba doubling time ± S.D. The points represent the 3 *D. discoideum* strains. The circles represent the 8 species of bacteria used in other parts of this study. Bacteria species identities by Brock et al. 2018 based on closest partial 16S BLAST hit are listed in Table 1.

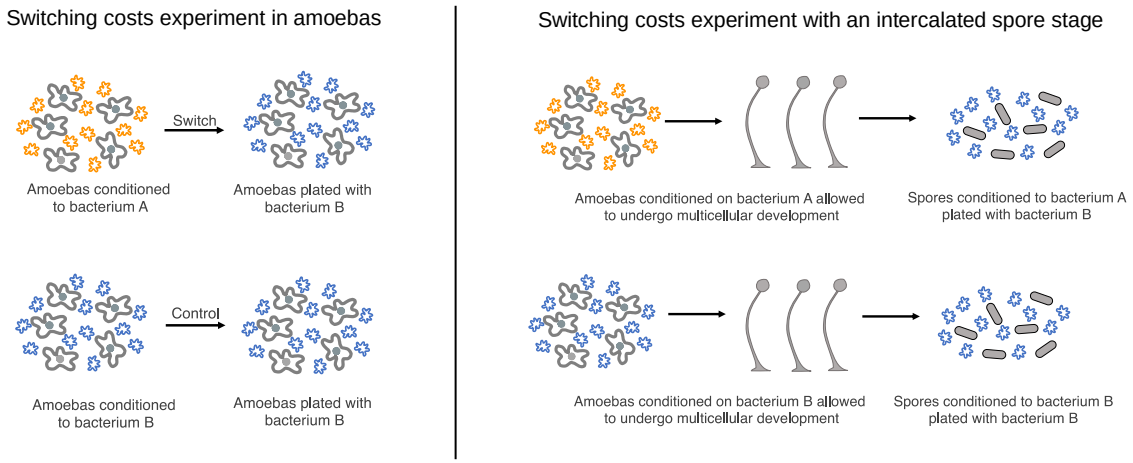
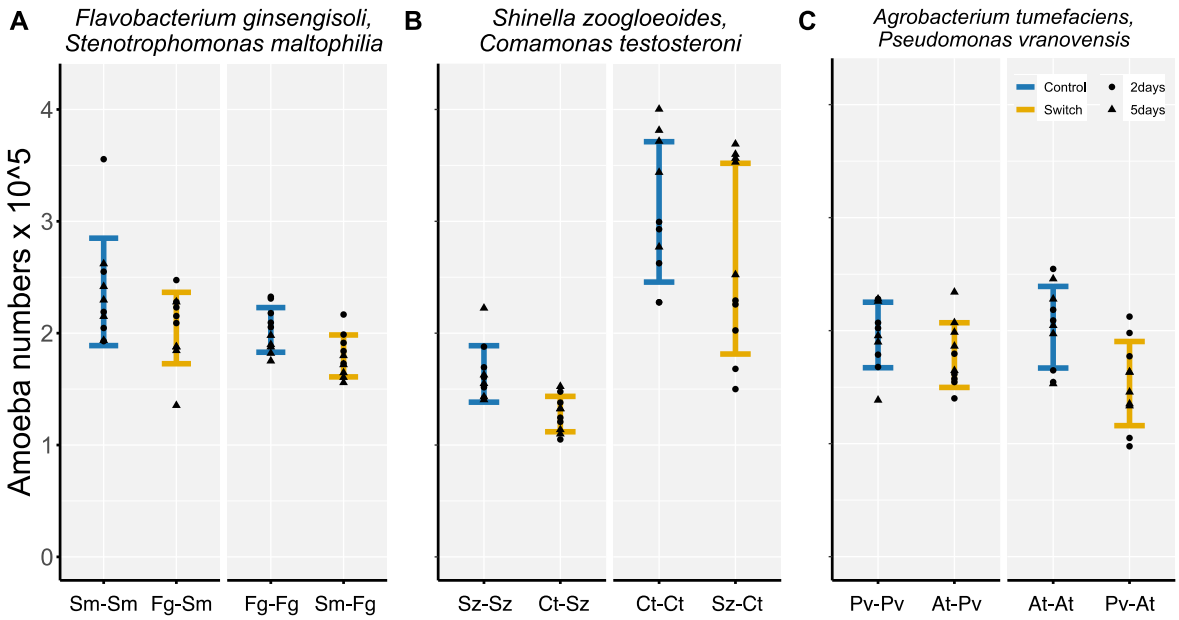


Figure 2. Schematic on the left shows switching costs experiment in amoebas. Amoebas in switched treatment are conditioned on bacterium A and are switched to bacterium B for the experiment. The controls for this experiment are amoebas conditioned to bacterium B plated with a fresh culture of the same. We also varied conditioning time of amoebas on a given bacterium (2 days, 5 days). Schematic on the right outlines switching costs experiment on spores. We used the same basic switching design as described for the amoebas, except we let the amoebas undergo multicellular development on the conditioning bacterium. We then collected spores from these fruiting bodies and performed the experiment.



559

560 **Figure 3:** *D. discoideum* amoebas experience prey switching costs: Mean amoeba numbers ± S.D

561 of controls and switches after 3 hours of growth. The shapes represent the different

562 conditioning times of amoebas (● - 2 days, ▲- 5 days). The points represent the five *D.*

563 *discoideum* strains. Total amoeba numbers are significantly different between controls and

564 switches pooled across all treatments ($F_{1,106}$ = 27.621, p-value = 7.69 x 10^{-7}). There was no

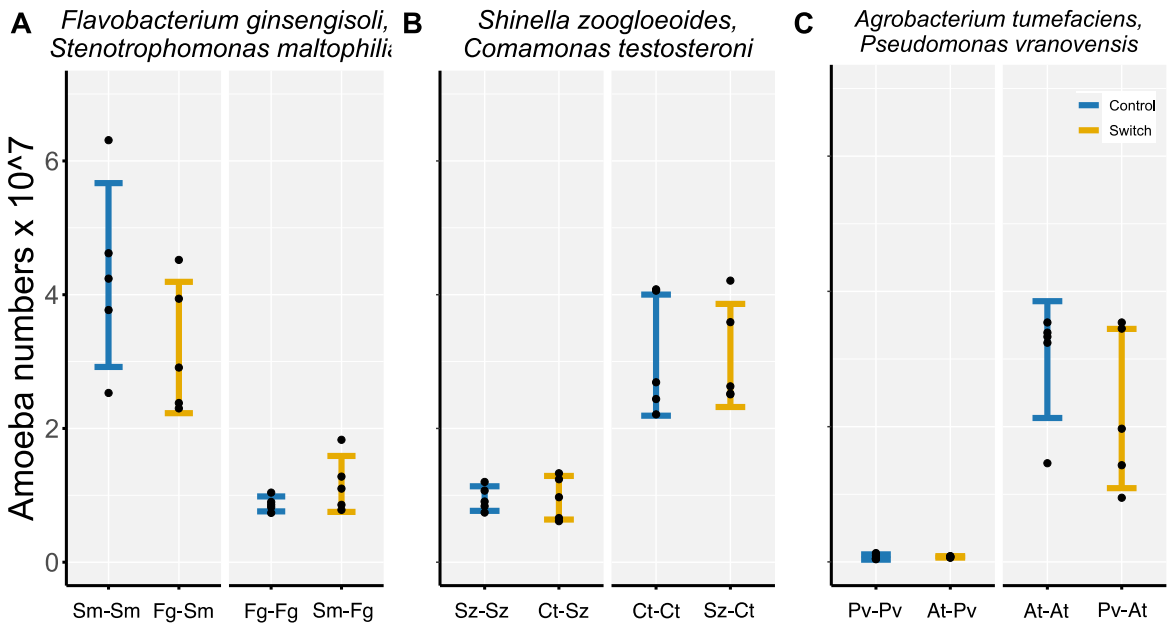
565 significant interaction between conditioning time and switching costs (Treatment x Conditioning

566 length: $F_{1,106}$ = 0.543, p-value = 0.463). Interpreting x-axis labels: For example - Sm-Sm refers to

567 amoebas grown on *S. maltophilia* moved to *S. maltophilia*, while Fg-Sm refers to amoebas

568 grown on *F. ginsengisoli* moved to *S. maltophilia*.

569



570

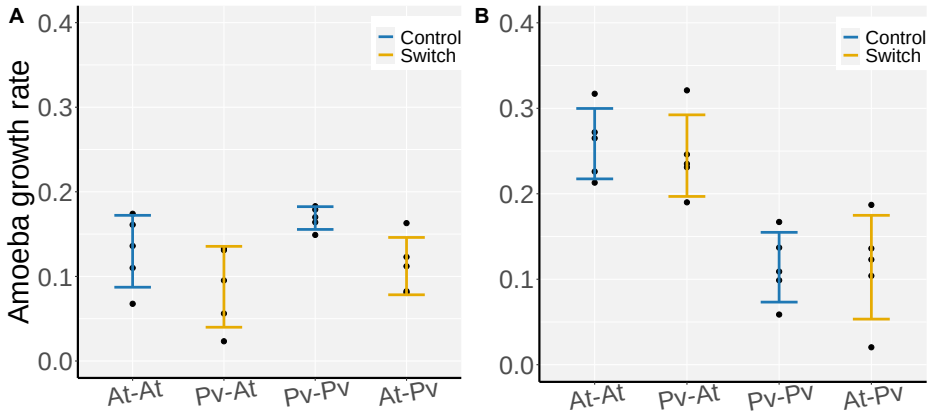
571 **Figure 4:** Switching costs disappear when spore formation precedes prey switch: Mean amoeba

572 numbers ± S.D of controls and switches at 36 hours from plating. The points represent the five

573 *D. discoideum* strains. We found no significant difference in amoeba numbers between controls

574 and switches ($F_{1,48}$ = 0.263, p-value = 0.61).

575



576

577 **Figure 5:** Switching costs occur during early growth period rather than later: A) Early growth rate

578 estimates of controls and switches between 0 and 6 hours from set-up. Switched treatments

579 have significantly lower early growth rates compared to controls $F_{1,17}$ = 9.458, p – value =0.007)

580 B) Late growth rate estimates of controls and switches between 24 to 27 hours from set-up for

581 (*Agrobacterium tumefaciens*, *Pseudomonas vranovensis*). We found no difference in growth

582 rates switches and controls at the later time points ($F_{1,17}$ = 0.111, p-value = 0.73) The points

583 represent 5 technical replicates of *D. discoideum* clone QS9.

584

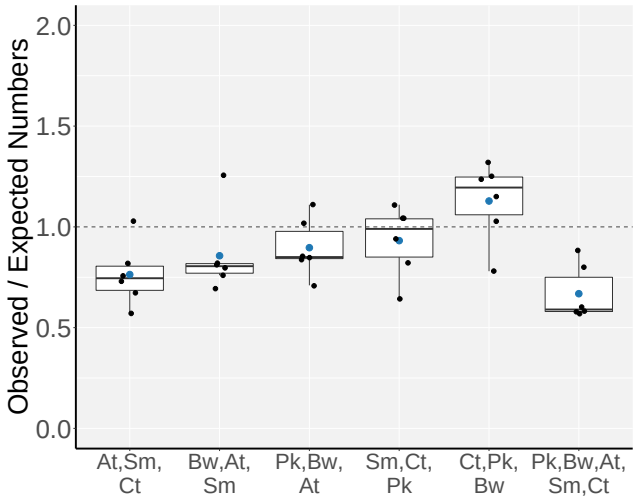


Figure 6: *D. discoideum* amoebas experience concurrent costs: Ratios of observed vs. expected amoeba numbers in different bacterial communities. A ratio lower than one indicates concurrent costs. Blue dot marks the mean. There are significantly fewer amoebas than expected in multi-species communities ($F_{1,59}$=11.83, p-value = 0.001). At- *Agrobacterium tumefaciens*, Bw- *Buttiauxella warmboldiae*, Ct - *Comamonas testosteroni*, Sm - *Stenotrophomonas maltophilia*, Pk - *Pseudomonas kuykendallii*.

593 **Table 1: List of 23 species of bacteria used in *D. discoideum* growth rate experiments. ***
594 **Species identification of bacterial isolates by Brock et al. 2018 based on closest partial 16S**
595 **BLAST hit.**

Strain number	Closest 16S sequence	Phylum/Class	Abbreviation
Lab clone	*Klebsiella pneumoniae*	Gammaproteobacteria	Kp
14P 8.1.1	*Pseudomonas kuykendallii*	Gammaproteobacteria	Pk
18P 8.2.2	*Buttiauxella warmboldiae*	Gammaproteobacteria	Bw
18P 2.2.1	*Agrobacterium tumefaciens*	Alphaproteobacteria	At
20P 9.1.2	*Agrobacterium rubi*	Alphaproteobacteria	Ar
14P 4.3.1	*Serratia liquefaciens*	Gammaproteobacteria	Sl
14P 6.2.3	*Stenotrophomonas maltophilia*	Gammaproteobacteria	Sm
18P 6.2.3	*Comamonas testosteroni*	Betaproteobacteria	Ct
20P 10.2.4	*Variovorax boronicumulans*	Betaproteobacteria	Vb
14P 8.1.4	*Comamonas kerstersii*	Betaproteobacteria	Ck
20P 3.2.2	*Flavobacterium ginsengisoli*	Bacteroidetes	Fg
20P 9.1.1	*Shinella zoogloeoides*	Alphaproteobacteria	Sz
20P 6.1.2	*Brucella papionis*	Alphaproteobacteria	Bp
5S 2.1.1	*Staphlycoccus saprophyticus*	Firmicutes	Ss
5P 5.1.1	*Pseudomonas vranovensis*	Gammaproteobacteria	Pv
18P 8.1.1	*Achromobacter aegrifaciens*	Betaproteobacteria	Aa
14P 5.3.2	*Pseudomonas migulae*	Gammaproteobacteria	Pm
20P 10.2.1	*Sphingobacterium ginsensidimutans*	Bacteroidetes	Sg
20P 2.1.2	*Pandoraea oxalativorans*	Betaproteobacteria	Po
14P 4.3.2	*Chryseobacterium rhizosphaerae*	Bacteroidetes	Cr
14P 6.2.1	*Microbacterium maritypicum*	Actinobacteria	Mm
20P 7.2.1	*Bacillus aryabhattai*	Firmicutes	Ba
20P 2.1.1	*Paenibacillus pabuli*	Firmicutes	Pp

596 **Supplemental information:**

597 **Table S1: ANOVA of amoeba growth rate assay**

598 **aov(Doubling_time ~ Test_bacterium)**

	Df	Sum sq	Mean Sq	F_value	Pr(>F)
Test_bacterium	22	299.346	13.3794	24.949	2.20E-16
Residuals	46	24.668	0.5363		

599

600 **Table S2: ANOVA of amoeba switching assay**

601 **aov(log(amoeba_number) ~ Treatment x Test_bacterium + Treatment x Conditioning_length)**

	Df	Sum sq	Mean Sq	F_value	Pr(>F)
Treatment	1	0.965	0.9648	27.621	7.69E-07
Test_bacterium	5	4.958	0.9917	28.392	2.00E-16
Conditioning_length	1	0.057	0.0567	1.623	0.205
Treatment x Test_bacterium	5	0.146	0.0293	0.838	0.526
Treatment x Conditioning_length	1	0.019	0.019	0.543	0.463
Residuals	106	3.702	0.0349		

602

603 **Table S3: ANOVA of spore switching assay**

604 **aov(log(amoeba_number) ~ Treatment x Test_bacterium)**

	Df	Sum sq	Mean Sq	F_value	Pr(>F)
Treatment	1	0.03	0.03	0.264	0.61
Test_bacterium	5	106.78	21.355	186.402	<2e-16
Treatment x Test_bacterium	5	0.68	0.136	1.191	0.328
Residuals	48	5.5	0.115		

605

606 **Table S4: ANOVA of early growth rate data**

aov (early.growth.rate ~ Treatment + Test_bacterium)

	Df	Sum_sq	Mean sq	F value	Pr(>F)
Treatment	1	0.012	0.012	9.458	0.007
Test_Bacterium	1	0.005	0.005	3.931	0.064
Residuals	17	0.022	0.001		

Table S5: ANOVA of late growth rate data

aov(late.growth.rate ~ Treatment + Test_bacterium)

	Df	Sum_sq	Mean sq	F value	Pr(>F)
Treatment	1	0.000	0.000	0.111	0.743
Bacteria	1	0.095	0.095	42.836	5.00E-06
Residuals	17	0.038	0.002		

Table S6: ANOVA of concurrent costs data

aov (log(Total.amoeba.number) ~ Treatment x Community + Experimenter)

	Df	SumSq	MeanSq	F-value	Pr(>F)
Treatment	1	0.4564	0.4564	11.8394	0.001071
Community	5	1.6847	0.3369	8.74	3.01E-06
Experimenter	1	24.3675	24.3675	632.0609	2.20E-16
Treatment x Community	5	0.4929	0.0986	2.5571	0.036767
Residuals	59	2.2746	0.0386		