Third-party effects in the *Dictyostelium-Paraburkholderia* symbiosis: food bacteria that are eaten, carried, or left behind

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Conflict of interest

We declare no conflicts of interest.

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Abstract

Symbiotic interactions change depending on the abundance of third parties like predators, prey, or pathogens. Third-party interactions with food bacteria are central to the symbiosis between *Dictyostelium discoideum* social amoeba hosts and inedible *Paraburkholderia* bacterial symbionts. Symbiosis with *Paraburkholderia* allows host *D. discoideum* to carry food bacteria through the dispersal stage where host amoebae aggregate and develop into fruiting bodies that disperse spores. Carrying bacteria benefits hosts when food bacteria are scarce but harms hosts when food bacteria are plentiful. The nature of this cost is unknown, but hosts leave bacteria behind when they carry symbionts. If this left-behind bacteria includes uneaten food bacteria, infected hosts may lose potential growth. Thus, decisions about how many food bacteria to eat, to carry, and to leave behind are crucial for understanding both benefits and costs in this symbiosis. We investigated how many food bacteria are uneaten and carried in this symbiosis by measuring fluorescently labeled food bacteria after fruiting body development. We found that *Paraburkholderia* infection makes hosts leave both symbionts and uneaten food bacteria but leaving food bacteria uneaten did not explain costs to hosts. Counts of food bacteria in fruiting bodies showed that hosts carry more food bacteria after developing in food-poor environments than in food-rich. This indicates that hosts, and possibly *Paraburkholderia* symbionts, actively modify how many food bacteria are carried to ensure hosts have food in the harshest conditions. Decisions about how many third-party bacteria to eat, carry, or leave may thus have important effects on this symbiosis.

Introduction

The fitness effects of symbiotic interactions can change depending on the environment (Bronstein, 1994; Chamberlain *et al.*, 2014; Horas *et al.*, 2022; Scott *et al.*, 2022). One crucial component of the environment can be a third species that interacts with hosts and symbionts (Palmer *et al.*, 2008; Wendling *et al.*, 2017; Wood *et al.*, 2018; Hafer-Hahmann & Vorburger, 2020; Cassidy *et al.*, 2022). For example, in the symbiosis between ants and *Acacia* plants, ants benefit *Acacia* by fending off herbivores. However, when herbivores were prevented from accessing *Acacias*, ordinarily mutualistic interactions between ants and *Acacias* shifted towards antagonism because ants no longer provided a benefit to host *Acacias* (Palmer *et al.*, 2008). Such shifts can influence whether host and symbiont fitness interests are aligned or in conflict (Keeling & McCutcheon, 2017; Iwai *et al.*, 2019). However, the details of how third parties affect symbioses are not well understood for many kinds of symbioses (Chamberlain *et al.*, 2014).

The social amoeba *Dictyostelium discoideum* is an important model of microbial symbiosis. *D. discoideum* is a soil amoeba that feeds on bacteria (Raper, 1937). Upon starvation, amoebae aggregate to form a multicellular fruiting body that disperses spores to new soil patches (Kessin, 2001). Development of the fruiting body progresses through well-studied stages starting with aggregation of individual amoebae which then become motile slugs (Bozzaro, 2019). Slugs can move to locations with food or find a location to develop into a fruiting body. During fruiting body development, about 20% of the cells die and become stalk while the remaining cells develop into spores that sit atop the stalk

in a structure called a sorus (Strassmann & Queller, 2011).

D. discoideum has symbioses with three species of facultatively intracellular *Paraburkholderia* bacteria (Brock *et al.*, 2020). In natural isolates, around 25% of wild-collected *D. discoideum* are infected by one, and occasionally two, of the three species of symbionts (Haselkorn *et al.*, 2019). When not referring specifically to one of the three species – *P. agricolaris*, *P. hayleyella*, or *P. bonniae* – we will refer to them collectively in this paper as either *Paraburkholderia* or symbionts. These symbionts are specialized for interacting with amoebae (Shu *et al.*, 2018a; b; Brock *et al.*, 2020). Symbionts move towards hosts (Shu *et al.*, 2018b) and make host phagosomes less acidic so that the symbionts are not digested (Tian *et al.*, 2022). One species of symbiont, *P. hayleyella,* appears to have led to host adaptation in response to the presence of symbionts (Shu *et al.*, 2018a). *P. hayleyella* and *P. bonniae* also have reduced genomes relative to *P. agricolaris* and most other *Paraburkholderia* (Brock *et al.*, 2020; Noh *et al.*, 2022), which indicates that *P. hayleyella* and *P. bonniea* have been associated closely with their hosts during a long evolutionary history (McCutcheon & Moran, 2012).

Fitness of *D. discoideum* hosts and *Paraburkholderia* symbionts is affected by interactions with a third set of organisms, the various food bacteria in the environment that are eaten by host amoebae (Brock *et al.*, 2011; DiSalvo *et al.*, 2015; Scott *et al.*, 2022). *Paraburkholderia* symbionts are largely inedible by hosts, and are carried undigested inside the sorus along with additional edible bacteria (Brock *et al.*, 2011; DiSalvo *et al.*, 2015; Khojandi *et al.*, 2019). When hosts disperse to food-poor environments where food bacteria are rare, hosts benefit from *Paraburkholderia* infection because hosts are able to carry food bacteria to seed new populations (Brock *et al.*, 2011;

DiSalvo *et al.*, 2015; Scott *et al.*, 2022). When infected hosts disperse to food-rich environments, hosts pay a cost of producing fewer spores relative to uninfected *D. discoideum* (Brock *et al.*, 2011; DiSalvo *et al.*, 2015). Presence of more *Paraburkholderia* is also associated with lower host spore production, but this effect depends on whether food bacteria are present (Scott *et al.*, 2022). Thus, host fitness depends on the abundance of food bacteria in the environment prior to fruiting body formation.

Fitness of inedible *Paraburkholderia* symbionts also changes depending on the density of edible bacteria that are eaten by hosts, probably because they compete for resources. We could thus refer to food-rich and food-poor host environments as being high and low competition environments for *Paraburkholderia* symbionts. To be consistent between host and symbiont environments, we will use food-rich and food-poor but we note that this affects competition for symbionts. After symbionts are carried to food-poor environments, both *P. agricolaris* and *P. hayleyella* reach higher densities relative to food-rich environments where they face more competition (Scott *et al.*, 2022).

The effect of food bacteria on hosts and symbionts highlights how food bacteria play an important role in this symbiosis. However, there are still many questions about how food bacteria affect this symbiosis throughout the development of *D. discoideum*. Of special interest is the period when fruiting bodies are formed and food bacteria are transferred into the sorus for carriage. This carriage period is important because the abundance of food bacteria impacts both parties (Brock *et al.*, 2011; DiSalvo *et al.*, 2015; Scott *et al.*, 2022). It is important to note that carrying food bacteria may be a joint phenotype (Queller, 2014; Queller & Strassmann, 2018) that both hosts and symbionts

affect together rather than being solely controlled by hosts. Joint phenotypes are interesting to study in symbioses because of the potential for cooperation and conflict over the value of the joint phenotype (Scott & Queller, 2019; Quides *et al.*, 2021). Here, it is the disposition of food bacteria that is of interest – should they be eaten, carried, or left behind?

Leaving food bacteria behind would not seem to be adaptive, but this appears to happen when hosts are infected by *Paraburkholderia* but not when they are uninfected (Brock *et al.*, 2011, 2016b). Initially, the key role of *Paraburkholderia* in causing hosts to leave behind bacteria was not known. It was thought that the bacteria left behind were all food bacteria (Brock *et al.*, 2011). This was taken as a possible sign of prudent predation by host amoebae — if some bacteria were going to be saved for carriage, hosts cannot eat all the available food and may stop feeding and start developing earlier (Brock *et al.*, 2011). It was hypothesized that hosts leaving food bacteria uneaten might explain why carriage is costly in some environments (Brock *et al.*, 2011).

The essential role of *Paraburkholderia* in the symbiosis, including causing hosts to leave behind bacteria is now known (DiSalvo *et al.*, 2015; Brock *et al.*, 2016b). Moreover, at least some of the bacteria left behind are not food bacteria but *Paraburkholderia* symbionts (Scott *et al.*, 2022). Because of this new information, the prudent predation hypothesis that hosts are leaving behind bacteria in order to carry should be re-evaluated. Several questions need to be clarified. What fraction of the bacteria left behind are food bacteria – how much real food is left on the table? Does *Paraburkholderia* infection cause hosts to leave the food bacteria uneaten? Are food bacteria left because the hosts cease eating and start developing earlier? What costs are

116 there for leaving food bacteria uneaten, or are costs instead due to the number of
117 *Paraburkholderia* symbionts?

118 While infected hosts may leave some food bacteria uneaten, they also gain the
119 ability to carry food bacteria along with dispersing spores in sori (Brock *et al.*, 2011;
120 DiSalvo *et al.*, 2015; Khojandi *et al.*, 2019). It is unknown whether the number of carried
121 food bacteria changes in different environments. Changes in the number of carried food
122 bacteria could depend on the abundance of food bacteria in the environment or on the
123 fitness interests of *D. discoideum* hosts and *Paraburkholderia* symbionts.

124 The number of carried food bacteria could reflect a passive process that mirrors
125 the densities of food bacteria in the environment. In this case, hosts that can carry (those
126 that are infected with *Paraburkholderia*) would carry food bacteria proportional to the
127 density of food bacteria in the environment. If this is the case, we expect hosts to carry
128 more food after developing in a food-rich environment and fewer food bacteria after
129 developing in a food-poor environment.

130 Alternatively, the number of carried food bacteria could reflect the evolutionary
131 interests of hosts and symbionts. Since soil environments tend to be spatially and
132 temporally structured (Sun *et al.*, 2003; Vos *et al.*, 2013), developing in a food-poor
133 environment may be associated with an increased probability that future environments
134 will also tend to be food-poor. If this is the case, hosts may carry more food bacteria
135 after developing in a food-poor environment than in a food-rich environment because
136 hosts that seed the next food-poor environment with food bacteria will have an
137 advantage. From the *Paraburkholderia* symbiont's perspective, allowing more carriage
138 of food bacteria would keep their hosts alive and allow further dispersal of symbionts by

139 hosts. Altering carriage in response to environmental conditions could therefore be
140 adaptive for both hosts and symbionts.
141 We investigate three questions about the role of food bacteria in the symbiosis
142 between *D. discoideum* and two commonly studied *Paraburkholderia* symbionts, *P.*
143 *agricolaris* and *P. hayleyella* (Shu *et al.*, 2018a; Garcia *et al.*, 2019; Scott *et al.*, 2022).
144 We first re-evaluate some of the ideas behind the prudent predation hypothesis by
145 measuring the density of any food bacteria that are left uneaten. We then test the fitness
146 consequences of prudent predation on hosts and ask if hosts that leave more food bacteria
147 uneaten pay a cost in spore production. Lastly, we turn to whether the number of food
148 bacteria carried inside sori changes between food-poor and food-rich environments.
149
150 **Methods**
151
152 *Clones and Culturing Methods*
153 To compare between infected and uninfected hosts, we used 12 host *D.*
154 *discoideum* clones, 4 clones uninfected, 4 infected with *P. agricolaris*, and 4 infected
155 with *P. hayleyella*. We refer to these infected clones as "reinfected" (Table 1) because
156 they were cured with tetracycline and reinfected with 0.1% of the *Paraburkholderia*
157 strain with which they were isolated. This reinfection scheme ensures that initial infection
158 densities were the same across clones.
159 We also used a set of uncured clones that have not been treated with antibiotics to
160 modify their native infection levels. For these clones, we used 7 clones naturally infected
161 with *P. agricolaris*, 7 clones naturally infected with *P. hayleyella*, and 6 clones that were

162 not infected with any *Paraburkholderia* symbionts (Table 1). One naturally uninfected
163 clone (QS1) was contaminated so we excluded it from the analysis leaving 6 clones in
164 our last infection category. These uncured clones were useful for checking whether our
165 results applied to unmanipulated infection densities.

166 To remove any effects of being in the freezer and to ensure that infected amoebae
167 carried fluorescently labeled *Klebsiella pneumoniae* food bacteria, we grew amoebae
168 through one round of feeding and fruiting body formation and then collected spores to
169 initiate our experiments. This step may allow infection densities time to equilibrate
170 (Miller *et al.*, 2020). Amoebae were grown from frozen spores at room temperature on
171 SM/5 plates (2 g glucose (Fisher Scientific), 2 g Bacto Peptone (Oxoid), 2 g yeast extract
172 (Oxoid), 0.2 g $MgSO_4$ * $7H_2O$ (Fisher Scientific), 1.9 g KH_2PO_4 (Sigma-Aldrich), 1 g
173 K_2HPO_4 (Fisher Scientific), and 15 g agar (Fisher Scientific) per liter) with 200 µL of
174 1.5 OD_{600} fluorescently labeled *K. pneumoniae* suspending in KK2 buffer (2.25 g
175 KH_2PO_4 (Sigma-Aldrich) and 0.67 g K_2HPO_4 (Fisher Scientific) per liter). *K.*
176 *pneumoniae* used in this study expressed green-fluorescent protein (GFP) and were
177 provided by dictyBase (Fey et al. 2019). To mimic dispersal to food-poor and food-rich
178 environments, we transferred 200uL of $2x10^5$/mL spore solution to plates with or without
179 200uL of 1.5 OD_{600} GFP-expressing *K. pneumonia*e. We let bacteria and amoebae on
180 plates proliferate at room temperature for six days, enough time for amoebae to form
181 fruiting bodies.

182

183 *Measurement of bacteria and host spore production*

184 To measure the density of uneaten food bacteria after *D. discoideum* fruiting body
185 formation, we first collected host spores and bacteria from plates after six days of growth
186 by washing plates with 15 mL of KK2 buffer. We counted host spores from this washed
187 solution using a hemocytometer. To measure bacterial density, we first removed host
188 spores by centrifuging the wash solution for three minutes at 1300 rpm and collecting the
189 supernatant. Removing host spores by manually removing sori with a pipette tip resulted
190 in similar densities of uneaten food bacteria suggesting that the number of bacteria inside
191 spores and sori is minimal relative to that left on the plate. After removing host material
192 by centrifugation, we measured the optical density of the supernatant at 600 nm (OD_{600})
193 as well as fluorescence with an excitation wavelength of 485 and emission wavelength of
194 515 nm in a 96 well plate with a Tecan Infinite 200 Pro microplate reader.
195 Since the washed plate solution from infected clones contains GFP-expressing *K.*
196 *pneumoniae* and unlabeled *Paraburkholderia*, the total OD_{600} is due to both kinds of
197 bacteria but fluorescence only comes from the *K. pneumoniae*. To calculate the amount
198 of OD_{600} due to fluorescing *K. pneumoniae*, we generated a standard curve relating
199 fluorescence to OD_{600} using serial dilutions of GFP-expressing *K. pneumoniae* in KK2
200 and used this curve to predict the OD_{600} of *K. pneumoniae* in our samples. The remaining
201 OD_{600} is the amount due to *Paraburkholderia* symbionts after subtracting off the
202 background OD_{600} of the KK2 buffer. An OD_{600} of 0.1 translates to around 5×10^7 *K.*
203 *pneumoniae* cells and 1×10^8 *Paraburkholderia* cells according to the validation dataset
204 in Scott et al. (2022).
205 To measure how many *K. pneumoniae* bacteria were carried in sori, we
206 haphazardly sampled a single sorus from each of our experimental plates from re-

207 infection experiments (n = 24). We suspended single sorus contents in KK2 buffer and
208 plated out serial dilutions. Since *K. pneumoniae* are labeled with GFP, we could
209 differentiate colonies of *K. pneumoniae* and *Paraburkholderi*a and get counts of colony
210 forming units (CFU). We then used these CFU counts to back-calculate the number of *K.*
211 *pneumoniae* bacteria inside fruiting bodies.
212
213 *Development assays for cured and reinfected hosts*
214 To determine how symbionts affected host development, we took time-lapse
215 images of cured and reinfected hosts growing in six well plates. We grew clones from the
216 freezer on SM/5 plates for six days in case there were freezer effects as above. We then
217 collected host spores and plated 30 uL of $2x10^5$ spores per mL and 30 uL of 1.5 OD *K.*
218 *pneumoniae* in each well. Photos were taken every hour until fruiting bodies developed
219 using a Canon EOS Mark IV. We inspected photos to determine time points for when
220 aggregates, slugs, and fruiting bodies first appeared in each well. We included three
221 replicates for each species and performed each individual replicate on a separate date.
222
223 *Calculating effect of uneaten food on host fitness*
224 To estimate how much host proliferation was lost by leaving food bacteria
225 uneaten, we used Kessin's (2001, p. 21) estimate that an amoeba is roughly 1,000 times
226 larger than a bacteria and would thus require around 1,000 bacteria to divide. To estimate
227 the number of food bacteria cells left uneaten, we used the validation data (Figure S1) in
228 Scott et al. (2022) that predicted *K. pneumoniae* colony forming units from OD_{600}. The

229 number of additional amoebae that would result from eating the uneaten food bacteria is
230 then the estimated number of food bacteria divided by 1,000.
231
232 *Statistical Methods*
233 To compare the density of *K. pneumoniae* bacteria left uneaten on the plate for
234 different infection categories (uninfected vs *P. agricolaris* infected vs *P. hayleyella*
235 infected and cured vs infected), we fit linear models in R (version 3.6.3). For uncured
236 infection comparisons that involved single measures of different clones, we used linear
237 models fit by generalized least squares (GLS) in the *nlme* package (Pinheiro & Bates,
238 2006). For reinfection experiments, which were performed on two separate dates with the
239 same clones, we fit linear mixed models (LMM) and included clone identity or date as a
240 random effect depending on which random effect resulted in a better fit. To compare
241 cured and infected hosts, we used ordinary linear regression with the *lm* function in R.
242 We performed Tukey post-hoc tests for pairwise comparisons using the *emmeans*
243 package (Lenth *et al.*, 2019).
244 To determine whether the timing of development was affected by
245 *Paraburkholderia* infection, we used generalized linear mixed models (GLMMs) with a
246 Poisson link function. We included the date that experiments were performed as a
247 random effect to capture variation within plates.
248 To determine how leaving food bacteria uneaten and *Paraburkholderia* infection
249 affect host spore production, we again used LMM and GLS for reinfections and uncured
250 infections, respectively. If prudent predation results in a cost for hosts, we expected a
251 decrease in fitness with increasing uneaten food bacteria. To test this, we fit linear models

252 of host spore production for infections with each species. Uninfected hosts' spore
253 production was included in each model to act as a baseline fitness when hosts are not
254 affected by infection. Differences in host fitness could also be affected by the density of
255 *Paraburkholderia* left on plates (Scott *et al.*, 2022) or by infection category (uninfected
256 vs infected) (Brock *et al.*, 2011; DiSalvo *et al.*, 2015; Haselkorn *et al.*, 2019). To account
257 for these possibilities, we also fit models with these variables along with a model that
258 includes both uneaten food density and *Paraburkholderia* density. In total, we compared
259 five models of spore production: (1) uneaten food density (Food model), (2)
260 Paraburkholderia density (Para model), (3) uneaten food density + Paraburkholderia
261 density (Food + Para model), (4) categorical infection status (Infection model), and (5) a
262 null model fit with only the intercept. To more easily compare effects across the different
263 models, we scaled all variables by subtracting the mean and dividing by the standard
264 deviation. We selected the best models among these five using AICc, a measure of model
265 fit that corrects for small sample sizes (Burnham & Anderson, 2004). Models that fit the
266 data best have lower AICc values.

267 To compare the number of *K. pneumoniae* inside fruiting bodies in food-rich and
268 food-poor environments, we used generalized mixed models with a logistic link function
269 to test whether the food environment affected the probability of having food in a sorus.
270 We transformed our response variable, the number of *K. pneumoniae* inside a fruiting
271 body, to a presence absence variable for the logistic regression. We used a logistic
272 function in this case because of the number of zeros in our data, which could cause
273 problems for linear mixed models and for generalized mixed models with Poisson fits.

274 We fit our model to data from both species but determined that species identity was not a
275 useful predictor using AICc.
276
277 **Results:**
278 *D. discoideum hosts infected with Paraburkholderia leave food bacteria uneaten*
279 We investigated the food bacteria left behind after hosts formed fruiting bodies by
280 measuring the density of leftover *K. pneumoniae* from cured and reinfected hosts and
281 naturally uninfected controls (Table 1). These densities are estimated from fluorescence
282 measurements since the *K. pneumoniae* used in this study expresses green fluorescent
283 protein (GFP; see methods). First, we wanted to confirm that some of the bacteria hosts
284 leave behind are food bacteria and not just *Paraburkholderia* symbionts. Leaving food
285 bacteria uneaten so that hosts miss out on potential growth and proliferation was a key
286 component of the prudent predation hypothesis, but previous studies did not differentiate
287 what kind of bacteria were left behind (Brock et al. 2011, 2016*b*). Hosts that were cured
288 and reinfected with *P. agricolaris* and hosts that were cured and reinfected with *P.*
289 *hayleyella* leave more food bacteria on the plate (Figure 1A) than naturally uninfected *D.*
290 *discoideum* (Tukey Post-Hoc test (TPH), $p \leq 0.001$ for both *Paraburkholderia* species).
291 This comparison supports the view that symbionts cause food bacteria to be left
292 uneaten but does not exclude the possibility that the differences are due to the different *D.*
293 *discoideum* clones that we used. Therefore, we next compared cured and reinfected hosts
294 to the same host genotypes that were cured of their native *Paraburkholderia* symbionts
295 but not reinfected. Both *P. agricolaris* ($p = 0.0139$) and *hayleyella* ($p = 0.0162$) re-

296 infections resulted in more food bacteria being left uneaten relative to cured but not
297 reinfected *P. agricolaris* and *hayleyella* hosts (Figure 1B).
298
299 *Host development is not affected by Paraburkholderia infection*
300 We hypothesized that leaving food bacteria uneaten would be associated with
301 faster development by infected hosts. To test this, we took time-lapse photos of amoebae
302 aggregating into fruiting bodies that either did or did not also carry *Paraburkholderia.*
303 We determined time courses for when they aggregated, formed slugs, and formed fruiting
304 bodies. We found that development times were not affected by *Paraburkholderia*
305 infection for either species (GLMMs; $p > 0.05$; Figure 2A&B).
306
307 *Food bacteria left on plates is not associated with reduced spore production in hosts;*
308 *Paraburkholderia infection is*
309 Leaving more food uneaten is suspected to lower host fitness and could therefore
310 explain the cost of *Paraburkholderia* infection relative to uninfected hosts (Brock *et al.*,
311 2011). Alternately, the cost of infection may be due to *Paraburkholderia* — this could be
312 measured as a categorical variable (infected vs uninfected) or as a continuous variable
313 (the density of *Paraburkholderia* symbionts on plates). Costs could also result from the
314 density of both uneaten food bacteria and *Paraburkholderia* symbionts.
315 We first tested the role of uneaten food bacteria and *Paraburkholderia* on host
316 spore production using our cured and reinfected hosts. For infections with both species of
317 *Paraburkholderia*, null models fit to only the intercept fit the data best (Figure 3A;
318 Supplemental File 1; the model that includes uneaten food bacteria and *Paraburkholderia*

319 density for *P. agricolaris* infected hosts — Food + Para — did show confidence intervals
320 that did not overlap zero, but this was the worst model in terms of AICc).
321 Our experiment with cured and reinfected hosts showed no effects of any of these
322 variables (Figure 3A) on host spore production (the null model without any variables was
323 the best fit). This includes no significant cost of infection, contrary to earlier results
324 (Brock *et al.*, 2011; DiSalvo *et al.*, 2015; Haselkorn *et al.*, 2019). The lack of cost may be
325 because hosts were infected with an extremely low density of *Paraburkholderia*
326 symbionts (Scott *et al.*, 2022). To test whether unmanipulated infection densities changed
327 our results, we performed a separate experiment with uncured hosts that have not been
328 treated with antibiotics (Table 1). Similar uncured clones have been shown to exhibit
329 costs of infection (Brock *et al.*, 2011).
330 Uncured hosts were afflicted by a cost of infection, but this cost was due to
331 *Paraburkholderia* infection instead of lost growth and proliferation from leaving uneaten
332 food bacteria. For uncured hosts with *P. agricolaris*, models of host spore production that
333 included uneaten food (Food model in Figure 3B), *Paraburkholderia* density (Para
334 model), and both variables together (Food + Para) were poor predictors of host spore
335 production (Supplemental File 1). The model with infection status was the best predictor
336 of host spore production for *P. agricolaris* uncured hosts (Supplemental File 1; note that
337 it is within 2 AICc units of the null model). These *P. agricolaris* results identify a cost of
338 infection like that found in prior studies (Brock *et al.*, 2011; DiSalvo *et al.*, 2015;
339 Haselkorn *et al.*, 2019).
340 For uncured hosts with *P. hayleyella*, we also found that *Paraburkholderia*
341 infection was responsible for costs. Uneaten food bacteria was associated with lower

342 spore production only if uneaten food was the only variable included in the model (Figure
343 3B). Including *Paraburkholderia* density changed the effect of uneaten food to slightly
344 positive with confidence intervals that overlap zero. *Paraburkholderia* density was a
345 better predictor of hosts spore production than uneaten food (Supplemental File 1) and
346 was highly correlated with uneaten food density (> 0.9). For uncured hosts infected with
347 *P. hayleyella*, infection status was again the best predictor (Supplemental File 1). Our
348 model comparisons for *P. hayleyella* also demonstrated a cost of infection. These models
349 of host spore production in uncured hosts point to a more direct role for
350 *Paraburkholderia* symbionts in reducing host spore production rather than a cost from
351 prudent predation.
352
353 *Uneaten food bacteria are a minority of left-behind bacteria*
354 Food bacteria left behind did not have the predicted effects on either development
355 time or spore production costs. One reason may be that the amount of left behind food is
356 not as great as formerly thought. In fact, uneaten food bacteria make up a minority of left-
357 behind bacteria, with the majority being *Paraburkholderia* (Figure 4). Using Kessin's
358 (2001, p. 21) rough estimate that an amoebae needs to eat 1,000 bacteria to divide, we
359 calculate that the number of uneaten food bacteria is only enough to produce 0.001-
360 0.004% more spores than what we recovered from plates. It is thus unlikely that uneaten
361 food bacteria can noticeably affect host fitness.
362
363 *More food bacteria are carried after hosts develop in food poor conditions*

364 To determine whether the number of food bacteria carried in sori simply reflects
365 the number of food bacteria in the previous environment or the interests of hosts and
366 symbionts, we measured the number of fluorescent food bacteria inside sori after growth
367 on food-rich and food-poor plates (Figure 5A). Counter-intuitively, we found that for
368 both *Paraburkholderia* species (Figure 5B&C), sori produced in food-rich environments
369 had a lower proportion of containing food bacteria (GLMM, Log odds = -1.894, se =
370 0.942, p = 0.044). This shows that the number of carried food bacteria is affected by food
371 context, but in the opposite direction to that expected if food was carried in proportion to
372 the density of food bacteria in the environment. Thus, the amount of carried food bacteria
373 may depend on the fitness interests of hosts and symbionts that benefit from carrying
374 more food bacteria when harsh conditions are expected.
375
376 **Discussion**
377 Third parties that interact with symbiotic partners can affect the fitness effects of
378 interactions (Palmer *et al.*, 2008; Chamberlain *et al.*, 2014; Wood *et al.*, 2018; Hafer-
379 Hahmann & Vorburger, 2020; Cassidy *et al.*, 2022). Often the details of how third parties
380 affect fitness effects are unknown. In the symbiosis between *D. discoideum* social
381 amoebae and *Paraburkholderia* bacteria, the third-party food bacteria is normally eaten
382 by the host but can also be carried or left behind. We studied how many food bacteria are
383 carried and left behind, and their impacts, by tracking fluorescently labeled food bacteria
384 (*K. pneumoniae*) during fruiting body formation by hosts.
385 Our most surprising finding was that hosts were more likely to carry food bacteria
386 after growing in food-poor environments (Figure 5). This result is surprising because it

387 means that hosts actively change the number of carried food bacteria depending on
388 environmental conditions. Since bacterial carriage is a joint phenotype affected by both
389 hosts and symbionts, *Paraburkholderia* symbionts may also play a role in modifying
390 carriage depending on the environment.

391 Our observation that food bacteria carriage depended on the environment (Figure
392 5) is possible evidence for mutual benefit between *D. discoideum* hosts and
393 *Paraburkholderia* symbionts. Host and symbionts are likely to benefit from modifying
394 carriage if future soil environments tend to resemble past soil environments Repeated
395 food-poor environments are where hosts should most benefit from carrying extra food
396 bacteria. *Paraburkholderia* symbionts could benefit from a stable association with hosts
397 that allows for continued dispersal, though more work is needed on the costs and benefits
398 of symbiosis for *Paraburkholderia* symbionts. We thus speculate that carrying more food
399 in these contexts may represent cooperation between hosts and symbionts that allows the
400 symbiosis to persist over repeated harsh environments. Such harsh conditions are
401 potentially an important force shaping this symbiosis (Scott *et al.*, 2022).

402 Questions remain about how hosts and symbionts affect the joint phenotype of
403 carrying food bacteria. *Paraburkholderia* are more often carried inside spores while food
404 bacteria are more often carried outside spores (Khojandi *et al.*, 2019), but the mechanism
405 for this is unknown. Hosts are able to normally modify the contents of fruiting bodies
406 through their immune cells that protect against toxins and bacteria by collecting potential
407 threats and dropping off during the slug stage (Chen *et al.*, 2007) but the role of these
408 immune cells play in the symbiosis (Brock *et al.*, 2016a) needs to be further explored.
409 Manipulation of phagosomes could also play a role in determining the contents of fruiting

410 bodies. *Paraburkholderia* symbionts increase the pH of phagosomes, presumably to
411 prevent host digestion of symbionts (Tian *et al.*, 2022). Similar modification of
412 lysosomes is used by pathogens to evade human immune cells during infection (Isberg *et*
413 *al.*, 2009; Leseigneur *et al.*, 2020). More work is needed to understand how symbionts
414 and food bacteria get into fruiting bodies and how both hosts and symbionts contribute to
415 bacterial carriage.

416 We also found that *Paraburkholderia* infection prevents hosts from eating all the
417 food bacteria in an environment (Figure 1). We expected this result given the role of
418 *Paraburkholderia* in causing hosts to carry food bacteria (DiSalvo *et al.*, 2015) and leave
419 behind bacteria (Brock *et al.*, 2016b; Scott *et al.*, 2022). Infection with *Paraburkholderia*
420 likely interferes with host digestion through modification of phagosomes (Tian *et al.*,
421 2022). This indigestion hypothesis is supported by our finding that *Paraburkholderia*
422 infection did not affect development time (Figure 2). Development time should be faster
423 for infected hosts since they appear to eat less food and should starve sooner. One
424 possible explanation is that *Paraburkholderia* symbionts are making hosts sick and
425 delaying development enough to result in no difference between uninfected and infected
426 hosts.

427 A prior study suggested that leaving food bacteria uneaten because of prudent
428 predation may explain the cost of infection relative to uninfected hosts (Brock *et al.*,
429 2011). We instead found that these costs to hosts were better explained by
430 *Paraburkholderia* infection than by the amount of food bacteria left behind (Figure 3).
431 The quantity of left behind food bacteria may be too small to noticeably affect host spore
432 production since we observed that only a minority of left-behind bacteria was food

433 bacteria, with the majority being indigestible *Paraburkholderia* (Figure 4). A rough
434 calculation of lost growth also showed that uneaten food bacteria was not enough to
435 substantially increase host fitness. We thus conclude that there is little support that
436 prudent predation explains the cost of infection in this symbiosis.

437 Instead of hosts paying a cost in potential growth because they leave food bacteria
438 uneaten, we suspect that *Paraburkholderia* infection causes both observations: that hosts
439 leave food uneaten and infected hosts pay a cost. Support for this idea comes from our
440 findings that *Paraburkholderia* density was correlated with uneaten food bacteria and
441 *Paraburkholderia* density was a better predictor of host fitness in *P. hayleyella* uncured
442 hosts (Figure 3). The role of *Paraburkholderia* in reducing host spore production is more
443 evidence in support of there being some conflict in this symbiosis (DiSalvo *et al.*, 2015;
444 Scott *et al.*, 2022).

445 Conflict may be more pronounced between *P. hayleyella* and its hosts than
446 between *P. agricolaris* and its hosts. We found that *P. hayleyella* density could explain
447 some of the decrease in uncured host spore production but did not find the same for *P.*
448 *agricolaris* density (Figure 3; Supplemental File 1). This difference between *P.*
449 *agricolaris* and *P. hayleyella* may result from *P. hayleyella* being more toxic than *P.*
450 *agricolaris* (Shu *et al.*, 2018a; Haselkorn *et al.*, 2019; Khojandi *et al.*, 2019). In addition
451 to being more toxic, *P. hayleyella* also has a reduced genome relative to *P. agricolaris*
452 (Brock *et al.*, 2020; Noh *et al.*, 2022). Reduced genomes are a common result of
453 persistent host association in beneficial symbionts and pathogens (McCutcheon & Moran,
454 2012). Since *P. hayleyella* maintains toxicity, it likely retains some pathogenic ability
455 against hosts.

456 An interesting remaining question in the *D. discoideum-Paraburkholderia*
457 symbiosis and in other symbioses is the mechanisms controlling conflict and causing
458 symbiont infection to reduce fitness. One potential explanation is that *Paraburkholderia*
459 symbionts directly feed on host cells or otherwise extract nutrients from hosts. Measures
460 of *Paraburkholderia* density inside sori have so far not been found to be correlated with
461 host fitness within species (Miller *et al.*, 2020), though the more toxic *P. hayleyella* does
462 appear to infect a higher percentage of spores than less toxic *P. agricolaris* (Shu *et al.*,
463 2018a; Khojandi *et al.*, 2019). Another promising hypothesis for the *D. discoideum-*
464 *Paraburkholderia* symbiosis that deserves further study is that hosts have lower spore
465 production because of "collateral damage" from competitive interactions between
466 *Paraburkholderia* and food bacteria (Scott *et al.*, 2022). Competition between bacteria is
467 often mediated by chemical warfare (Granato *et al.*, 2019) that could reduce host *D.*
468 *discoideum* fitness as a side-effect.
469 Whether a specific symbiosis involves fitness alignment or conflict may depend
470 on a third party that affects the costs and benefits of symbiosis. Our results show that
471 third parties can have complex effects on symbioses; the symbiosis between *D.*
472 *discoideum* and *Paraburkholderia* appears to involve elements of conflict and
473 cooperation that are affected in multiple ways by food bacteria.
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591 **Figure and Table Legends**

592 Table 1: Clones used in experiments.

Type of infection	Description	*Paraburkholderia* Treatment	Clones
Cured and reinfected	Clones that were cured of any native symbionts with tetracyline and reinfected in the lab with 0.1% of their native symbiont	*P. agricolaris* infected	QS159, QS161, QS606, NC21
		P. hayleyella infected	QS395, QS45, QS38, QS23
		Uninfected control	QS6, QS138, QS472, QS527
Uncured	Clones with or without natural infections that have	*P. agricolaris* infected	QS494, QS756, QS788, QS113, QS453, QS70, QS606

	not been treated with antibiotics	*P. hayleyella* infected	QS45, QS101, QS46, QS2, QS23, QS529, QS38
		Uninfected control	QS4, QS6, QS14, QS18, QS9, QS8

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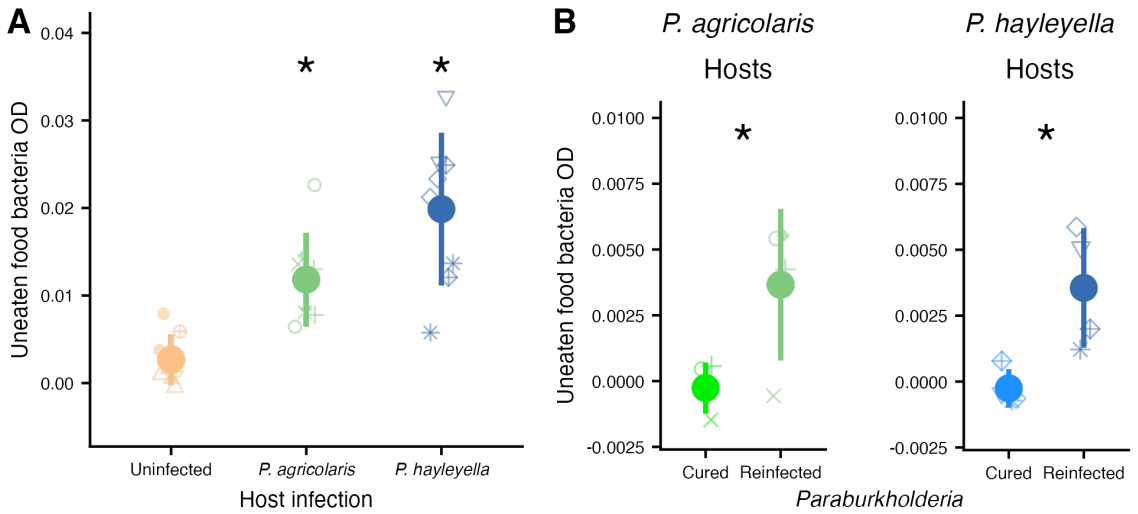

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599 **Figure 1:** Symbionts cause hosts to leave food uneaten. (A) Density left on plate of

600 uneaten *K. pneumoniae* food bacteria (measured by OD_{600}) for naturally uninfected, *P.*

601 *agricolaris* cured and reinfected, and *P. hayleyella* cured and reinfected hosts. Reinfected

602 hosts were reinfected with their natural symbiont partners. (B) Density left on plate of

603 uneaten food bacteria for naturally infected *P. agricolaris* and *P. hayleyella* hosts that

604 were either cured or reinfected. Shapes indicate clones (see Figure 1A in Scott et al.

605 2022). Dot and lines show mean and standard deviation, respectively. Asterisks indicate

606 significant differences (in panel A comparisons are between infected and uninfected).

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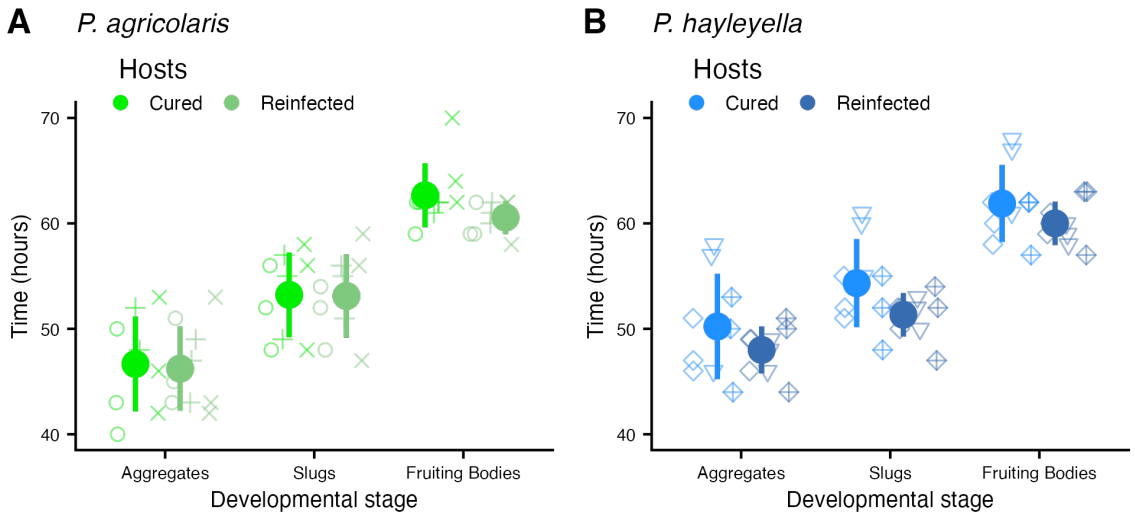

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609 **Figure 2**: Symbionts do not affect development. Developmental time points for cured

610 and reinfected (A) *P. agricolaris* and (B) *P. hayleyella* hosts at different stages of

611 development. Shapes indicate clones (see Figure 1A in Scott et al. 2022). Dot and lines

612 show mean and standard deviation, respectively.

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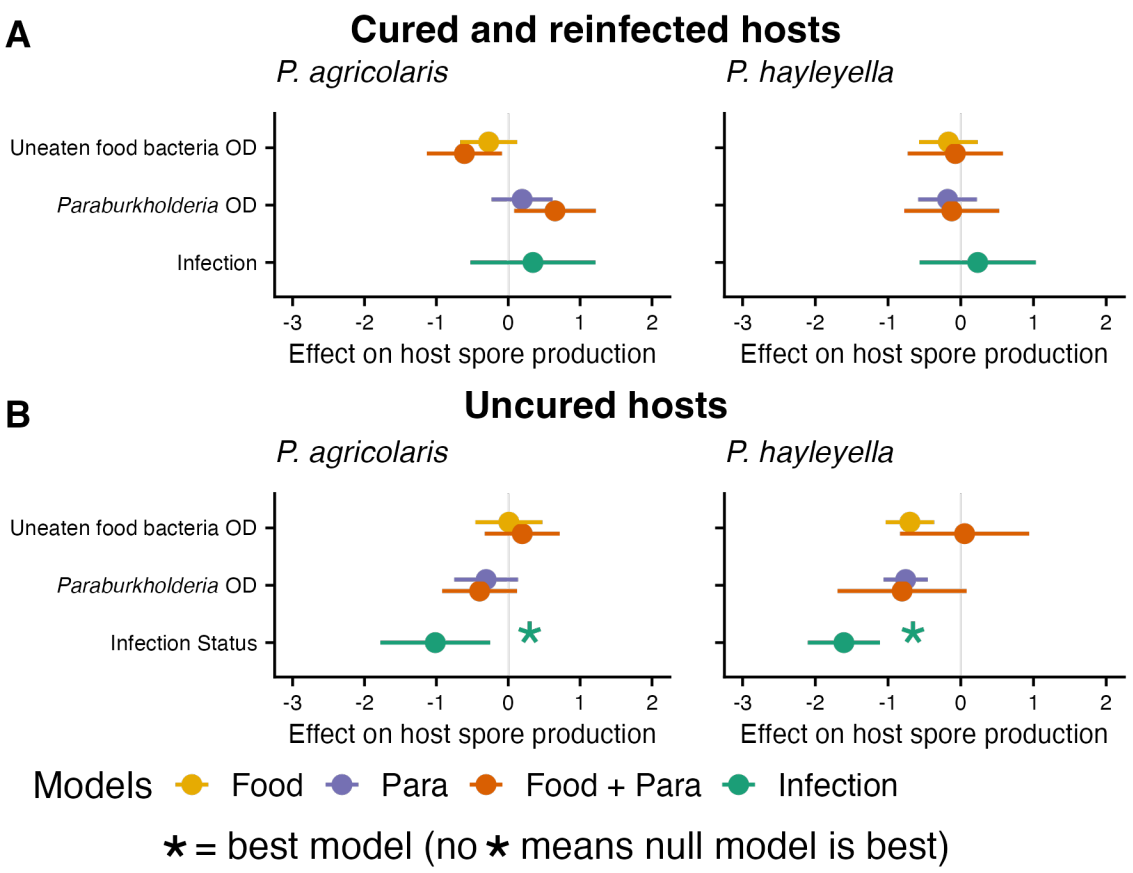
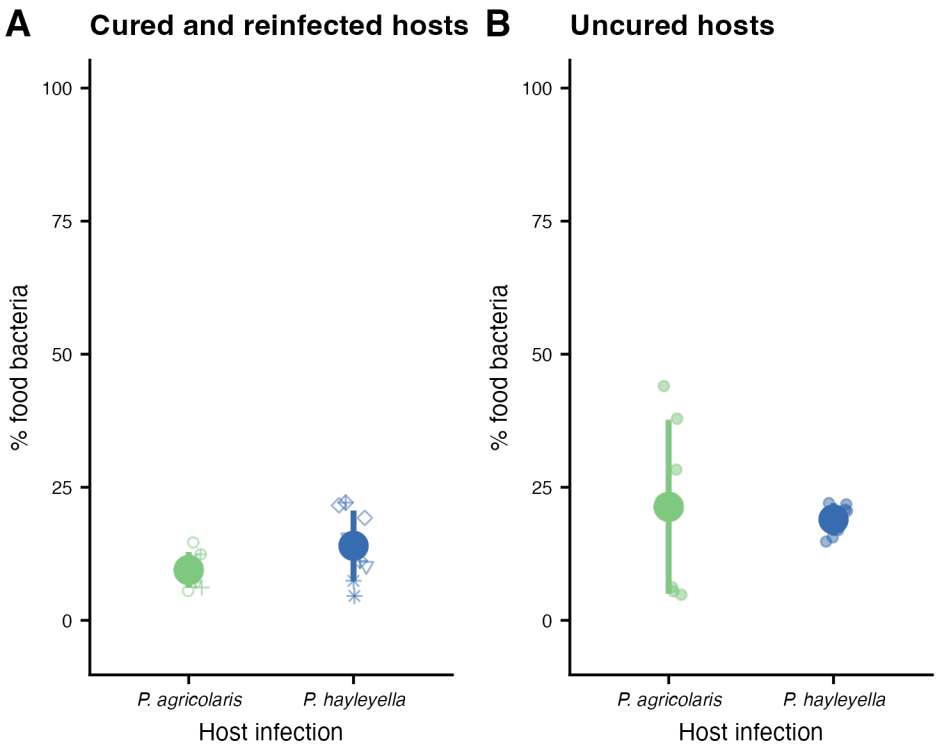


Figure 3: Estimated effects of bacteria left on plates and infection status on host spore production in (A) cured and reinfected (B) and uncured hosts (see Table 1). Hosts without *Paraburkholderia* are included as baselines in all models. We compared models of host spore production (shown in different colors), predicted by *Paraburkholderia* density only (OD), uneaten food bacteria only (OD), both *Paraburkholderia* and uneaten food bacteria, or a categorical variable for infection status (infected with *Paraburkholderia* or not). Estimated effects are shown as points and 85% confidence intervals are shown as lines (null models with only the intercept are not shown). Asterisk color indicates the best model according to AICc (there is no asterisk in A because the null models fit with only the intercept are the best models).



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628 **Figure 4:** Most of the bacteria left behind by hosts were not food bacteria. Percent of the
629 total left behind bacteria (includes food bacteria and Paraburkholderia symbionts) that
630 was food bacteria from cured and reinfected (A) and uncured (B) hosts. Shapes in A
631 show host clone replicates that were measured on two separate dates (see Figure 1A in
632 Scott et al. 2022). Points in B are independent, so we do not show clone identity. Dot and
633 lines show mean and standard deviation, respectively.

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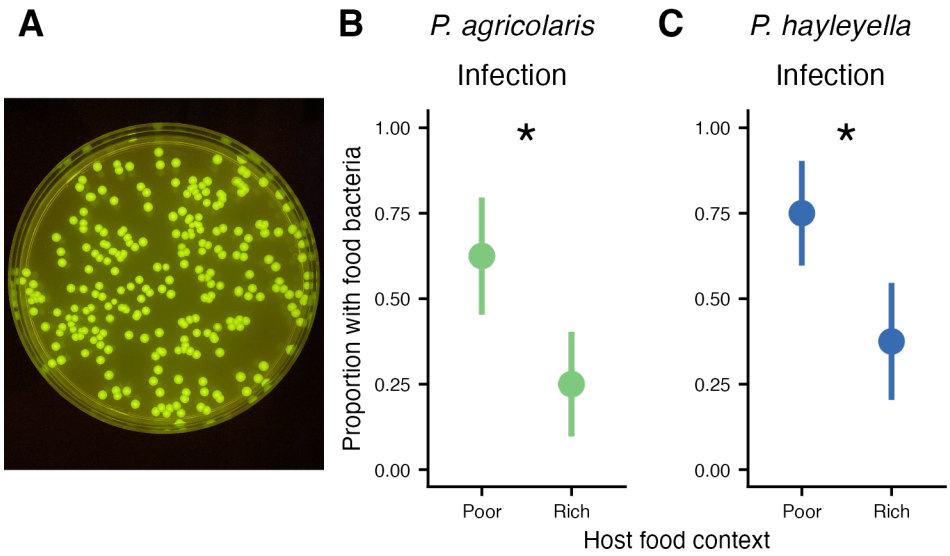

Figure 5: Hosts are less likely to carry food bacteria in sori after developing in food-rich environments. (A) Photo of fluorescent *K. pneumoniae* food bacteria colonies plated from an individual sorus. (B) Proportion of sori with carried food bacteria for reinfected *P. agricolaris* hosts from food-poor and food-rich contexts. (C) Proportion of sori with carried food bacteria for reinfected *P. hayleyella* hosts from food-poor and food-rich contexts. Points show proportions and lines show the standard deviation. Asterisks indicate significant differences (GLMM).