Winter 12-15-2022

Cooperation and Conflict in the Social Amoeba Dictyostelium discoideum and its Paraburkholderia Endosymbionts

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Cooperation and Conflict in the Social Amoeba *Dictyostelium discoideum* and its *Paraburkholderia* Endosymbionts

by
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A dissertation presented to
Washington University in St. Louis
in partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

December 2022
St. Louis, Missouri

Table of Contents

List of Figures....iv
List of Tables....vi
Acknowledgments....vii
Abstract....viii
Chapter 1: Cooperation and Conflict in the Social Amoeba *Dictyostelium discoideum*....1
1.1 Abstract....1
1.2 Introduction....2
1.3 Benefits of the social cycle....5
1.4 Relatedness in nature and how it is generated....6
1.5 Costs and benefits of associating with non-relatives....8
1.6 Genes for cheating....11
1.7 Power....12
1.8 Cooperation is maintained by control of cheaters....14
1.9 Relevance of cooperation and cheating for *D. discoideum*....18
1.10 Other domains of cooperation and conflict....21
1.11 Conclusion....24
Chapter 2: Limits to the Spread of an Obligate Social Cheater in *Dictyostelium discoideum*....41
2.1 Abstract....41
2.2 Introduction....41
2.3 Methods....45
2.4 Results....50
2.5 Discussion....51
2.6 References....54
2.7 Figures....57
Chapter 3: The Social Amoeba *Dictyostelium discoideum* rescues *Paraburkholderia hayleyella* but not *P. agricolaris* from interspecific competition....60
3.1 Abstract....60
3.2 Introduction....61

3.3 Methods 65
3.4 Results 68
3.5 Discussion 70
3.6 References 74
3.7 Figures 77
Chapter 4: Kin discrimination and competition in bacterial endosymbionts of the social amoeba *Dictyostelium discoideum*, *Paraburkholderia agricolaris* and *P. hayleyella* 90
4.1 Abstract 90
4.2 Introduction 90
4.2 Materials and methods 95
4.3 Results 97
4.4 Discussion 100
4.5 References 104
4.6 Figures 109

List of Figures

Figure 1.1: Social cycle of *D. discoideum*.....32

Figure 1.2: *D. discoideum* relatedness in nature.....33

Figure 1.3: Genetic demixing in *D. discoideum*.....34

Figure 1.4: Allorecognition in Dictyostelids.....35

Figure 1.5: Costs of chimerism in *D. discoideum*.....36

Figure 1.6: Cheating strategies in *D. discoideum*.....37

Figure 1.7: Power impacts cell fate in *D. discoideum*.....38

Figure 1.8: Relatedness affects evolution of cheaters in *D. discoideum*.....39

Figure 1.9: The sexual cycle of *D. discoideum*.....41

Figure 2.1: Initial cheater proportion predicts stalk height.....58

Figure 2.2: Initial cheater proportion predicts final cheater proportion.....59

Figure 3.1: Competition with *K. pneumoniae* harms both *P. hayleyella* and *P. agricolaris*.....77

Figure 3.2: *D. discoideum* rescues some clones of *P. hayleyella* and *P. agricolaris*.....78

Figure 3.3: *P. hayleyella* is harmed more than *P. agricolaris*.....79

Figure 3.4: Variation in rescue effect of *D. discoideum* across strains of *P. agricolaris*.....80

Figure 3.5: Variation in rescue effect of *D. discoideum* across strains of *P. hayleyella*.....81

Figure 3.6: *P. agricolaris* is harmed competition with *K. pneumoniae*.....82

Figure 3.7: Variation in rescue effect of *D. discoideum* across strains of *P. agricolaris*.....83

Figure 4.1a: Example growth curves with phage.....110

Figure 4.1b: Example growth curves without phage.....111

Figure 4.2: Example swimming assay for *P. agricolaris*.....112

Figure 4.3: Example swimming assay for *P. hayleyella*.....113

Figure 4.4: Example agar diffusion assay for *P. hayleyella*.....114

Figure 4.5: Example agar diffusion assay for *P. agricolaris*.....115

Figure 4.6: Bacteriophage assay.....116

Figure 4.7: Growth rate of mixed cultures.....117

Figure 4.8: Carrying capacity of mixed cultures.....118

Figure 4.9: Lag time of mixed cultures.....119

List of Tables

Table 3.1: Parameters from generalized linear models 5:95.....87
Table 3.2: Parameters from generalized linear models 50:50.....89
Table 4.1. Results of assays of kin discrimination.....112

Acknowledgments

I would like to acknowledge my co-authors that contributed to this work: Joan Strassmann, David Queller, Justine Garcia, P.M. Shreenidhi, and Tyler Larsen.

I would like to thank my advisors Joan Strassmann and David Queller for their support and mentorship.

I would also like to thank the members of my committee, past and current members of the Queller-Strassmann lab, and the other graduate students in EEPB for their help and support.

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December 2022

ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Biology and Biomedical Sciences

Evolution, Ecology and Population Biology

Washington University in St. Louis, 2022

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A big question in biology is how organisms compete in an environment of competitors for scarce resources. Part of the answer lies in distinguishing friend from foe and in forging cooperative bonds in the face of cheaters. The social amoeba – bacteria system I have studied here is an excellent place to explore these tensions. The first part of my thesis research involves a review of cooperation and conflict in the social amoeba *Dictyostelium discoideum* and a study of the limits on obligate social cheating in this species. The second part focuses on the benefits of associating with *D. discoideum* for two bacterial intracellular endosymbionts, *Paraburkholderia agricolaris* and *P. hayleyella.* I also explore kin discrimination within each of these species. I found that an obligate social cheater in *D. discoideum* is limited by negative frequency-dependent cheating and reduced potential for dispersal. One endosymbiont that I studied, *P. hayleyella,* has a reduced genome and is more AT rich than non-symbiotic congeners, similar to obligate endosymbionts. I found that this endosymbiont benefits from *D. discoideum* in the context of interspecific resource

competition while the species more similar to non-symbiont species, *P. agricolaris*, does not get this benefit. I found surprisingly little kin discrimination between strains of *P. agricolaris* and *P. hayleyella*.

Chapter 1: Cooperation and Conflict in the Social Amoeba *Dictyostelium discoideum*

James M. Medina, P.M. Shreenidhi, Tyler J. Larsen, David C. Queller, Joan E. Strassmann

1.1 Abstract

The social amoeba *Dictyostelium discoideum* has provided considerable insight into the evolution of cooperation and conflict. Under starvation, *D. discoideum* amoebas cooperate to form a fruiting body comprised of hardy spores atop a stalk. The stalk development is altruistic because stalk cells die to aid spore dispersal. The high relatedness of cells in fruiting bodies in nature implies that this altruism often benefits relatives. However, since the fruiting body forms through aggregation there is potential for non-relatives to join the aggregate and create conflict over spore and stalk fates. Cheating is common in chimeras of social amoebas, where one genotype often takes advantage of the other and makes more spores. This social conflict is a significant force in nature as indicated by rapid rates of adaptive evolution in genes involved in cheating and its resistance. However, cheating can be prevented by high relatedness, allorecognition via *tgr* genes, pleiotropy, and evolved resistance. Future avenues for the study of cooperation and conflict in *D. discoideum* include the sexual cycle as well as the relationship between *D. discoideum* and its bacterial symbionts. *D. discoideum's* tractability in the laboratory as well as its uncommon mode of aggregative multicellularity have established it as a promising model for future studies of cooperation and conflict.

1.2 Introduction

The study of cooperation and conflict among living organisms has traditionally focused on the behavior of social animals like ants, lions, or primates, but the central ideas apply to all life. Cooperation and conflict are of great evolutionary importance even to organisms with no intelligence and no behavior in the conventional sense. One microbe – the social amoeba *Dictyostelium discoideum* – has in recent decades taken a special significance for scientists seeking to understand how cooperation and conflict evolve. Its tractability in laboratory studies, its long history as a model for studying development and immunology, and most importantly its unusual life cycle make it particularly useful (Kaushik and Nanjundiah, 2003; Kessin, 2001; Li and Purugganan, 2011; Ostrowski, 2019; Shaulsky and Kessin, 2007; Strassmann and Queller, 2011).

D. discoideum and its relatives are cellular slime molds found in soils throughout the world (Swanson *et al.*, 1999). *D. discoideum* spends most of its life as a single-celled, vegetative amoeba, traveling through the soil and preying upon bacteria. As bacterial prey are depleted and the amoebas begin to starve, *D. discoideum* enters a unique social cycle (Fig 1.1). Previously solitary cells rapidly transition to multicellularity, aggregating into a slug-like multicellular body of tens to hundreds of thousands of cells. The slug migrates to a suitable spot and matures into a fruiting body, its constituent cells developing into a sorus of durable spores which can wait dormant for conditions to improve and a tall stalk to hold the sorus aloft and increase the spores' chances of being dispersed by a passing invertebrate (Bonner, 1967; Kessin, 2001; smith *et al.*, 2014). Crucially, the development of the stalk is an act of altruism by stalk cells, which die in the process of helping spore cells survive and disperse.

Therein lies a problem. Natural selection should only select for adaptations that increase the reproductive success of individuals carrying the genes underlying them. Cells that die to produce the fruiting body's stalk cannot pass on their genes to the next generation. Only spores – those cells which did *not* sacrifice themselves – live to produce progeny. Stalk cells pay a price and seem to receive nothing in return. Why, then, does natural selection not eliminate stalk production altogether? Why do 'cheaters' – strains which abandon or reduce their investment in stalk production to take advantage of the stalks produced by cooperators – not rapidly overtake the population?

The self-sacrifice of *Dictyostelium* stalk cells is analogous to the sterility of social insect workers, the suicidal stinging defense of honeybees, the risky cooperative hunting of larger prey by pack hunting mammals, and myriad other examples with the same potential evolutionary pitfalls. Why should any organism evolve a capacity to sacrifice its own reproduction for the reproduction of others? This is the problem of altruism, and it is one of the historic puzzles in evolutionary biology. Altruistic traits should be evolutionarily unstable and yet such traits appear throughout nature.

An important answer arrived in the 1960s when William Hamilton quantitatively formalized a general explanation for the evolution of apparently altruistic behaviors called inclusive fitness theory or kin selection theory (Hamilton, 1964a; Hamilton, 1964b). Under inclusive fitness theory, natural selection acts on individuals' *inclusive fitness*, which consists of both their personal or direct fitness – their lifetime reproductive success – as well as any fitness obtained through their effects on genetic relatives. By helping close relatives reproduce, individuals can indirectly transmit copies of their genes to the next generation. Selection on benefits to kin can thus even select for extreme altruistic traits (like stalk production in *D. discoideum)* where some

individuals sacrifice themselves entirely for their kin. Inclusive fitness theory has proven to have a great deal of explanatory power, not only in justifying the existence of altruism, but also for predicting phenomena like worker policing and extreme sex ratios in social insect colonies (Bourke, 2011; Bourke and Franks, 1995; Queller, 2016; Ratnieks *et al.*, 2006; Strassmann *et al.*, 2011).

Inclusive fitness theory does much to explain why *D. discoideum* might retain self-sacrificial traits like stalk formation. In many fruiting bodies, just as in the bodies of more conventional multicellular organisms like animals, most or all of the constituent cells will be clones, and as such any gene present in a would-be stalk cell is very likely to be present in the spore cell the stalk cell's self-sacrifice would benefit. If fruiting bodies were all clonal, the costs of a subset of cells dying to produce a stalk could be compensated for by the dispersal and/or survival benefits afforded to the rest of the cells. Major questions remain, however. Even in a clonal organism, mutation can produce new variation and reduce relatedness (though this appears not to be a major problem in *D. discoideum* (Kuzdzal-Fick *et al.*, 2011)). More importantly – and unlike most other multicellular organisms – *D. discoideum* forms its multicellular body via the aggregation of all nearby cells, whether they are clonemates or not. This opens opportunities for fruiting bodies to have relatedness much lower than one, and thus for the evolution of conflict and the disruption of cooperation.

D. discoideum and its relatives have persisted in performing their social life cycles in the face of potential conflict and evolutionary instability, so it stands to reason that they must have ways to mitigate the risk these factors pose. But what are these mitigating factors? What are the costs and benefits of cooperating or cheating in nature? What Dictyostelid traits may have been preadaptations that made it robust against cheaters from the start and allowed it to evolve its

cooperative lifestyle? What adaptations may have evolved after the fact to control, exclude, or eliminate cheaters?

These questions and their answers are the focus of this review.

1.3 Benefits of the social cycle

D. discoideum's social stage requires the death of ~20% of the cells, but there are many benefits that compensate for this cost. When starved, amoebas aggregate into a motile slug. Slugs can move much farther than individual cells can on their own and cross gaps in the soil that amoebas could not (Kuzdzal-Fick *et al.*, 2007). The slug stage thus helps *D. discoideum* aggregates find suitable environments to form fruiting bodies (Kessin, 2001), and by sloughing off cells in its wake, may also facilitate dispersal into new areas (Kuzdzal-Fick *et al.*, 2007). The slime sheath secreted during slug formation and migration can also protect the amoebas from predation by nematodes (Kessin *et al*., 1996). Once *D. discoideum* forms a fruiting body and produces a stalk, spores are held aloft where they are more likely to be dispersed to new environments via animal vectors such as small invertebrates like pillbugs, earthworms and nematodes (Huss, 1989; Kessin *et al*., 1996) or vertebrates like ground-feeding birds, small rodents, salamanders, and bats (Stephenson and Landolt, 1992; Suthers, 1985). Lab studies using *Drosophila* as a model arthropod vector show that spores are dispersed more effectively when fruiting bodies are intact compared to when they are experimentally knocked over (smith *et al.*, 2014). Alternatively or in addition to dispersal, the stalk may lift the spores above the hazards of the soil (Bonner, 1982; Kessin, 2001). Finally, the social cycle's spore production is clearly beneficial because spores can resist harsh environmental conditions such as long periods of cold, heat, or drought, as well as digestion by animals (Raper, 1984).

1.4 Relatedness in nature and how it is generated

For an altruistic act to evolve, it must confer benefits to relatives. The social cycle in *D. discoideum* is altruistic and has clear benefits, but do the benefits go to relatives? To answer this we need to know the relatedness among *D. discoideum* cells within the same fruiting body in nature. Genetic relatedness is the probability above random expectation that an allele found in one individual is present in another (not, as is sometimes mistakenly assumed, a measure of overall fraction of shared genes.) A relatedness of 0 indicates random mixing and a relatedness of 1 indicates perfect assortment into genetically uniform fruiting bodies. For altruism to evolve, it is necessary (but not sufficient) that relatedness to beneficiaries must be well above zero. In some Dictyostelids like *D. purpureum* and *D. giganteum* there is evidence that co-occurrence of different genotypes in the same fruiting body in nature can occur (Sathe *et al.*, 2010). In *D. discoideum*, by contrast, relatedness within fruiting bodies found in nature has been estimated using neutral microsatellite markers to be quite high, averaging between 0.86 and 0.975 (Gilbert *et al.*, 2007). This high relatedness could be generated in several different ways.

One way that high relatedness can be generated is through spatial structure. If clonal patches of amoebas are typically far enough apart from one another that they do not generally aggregate with cells of other genotypes, then fruiting bodies will usually be clonal (Fig 1.2). In fact, when patches are initiated from single cells, only a few millimeters of distance is required to generate high relatedness within fruiting bodies in *D. discoideum*. Furthermore, even adjacent fruiting bodies can be different genotypes (smith *et al.*, 2016). This kind of structure, where patches grow up from single cells and do not mix much, is similar to the single-cell bottlenecks that initiate more conventional multicellular organisms. However, the extreme of a single cell bottleneck is not necessary to generate high relatedness if close relatives disperse together as a group (Gardner

and West, 2006; Inglis *et al.*, 2017; Queller and Strassmann, 2012). This may be the case in *D. discoideum* due to its sticky spores that could stick together through dispersal.

Relatedness can also be raised by structured population growth from a genetically mixed group of cells through a process called genetic demixing (Fig 1.3) (Queller and Strassmann, 2012). Most outward growth will be from cells at the edge of the group and each sector of the edge will contain few enough cells that random drift can determine which genotype succeeds in that sector. As cells divide and give rise to their neighbors, they form sectors of different genotypes. This phenomenon is well known from bacteria (Gralka *et al.*, 2016; Hallatschek *et al.*, 2007; Hallatschek and Nelson, 2010), and though extensive movement of amoebas might be expected to prevent it, it has been observed in *D. discoideum* grown on agar as well (Buttery *et al.*, 2012). It remains to be determined if this process also occurs in the more natural environment of soil, and if spatial growth of *D. discoideum* in the vegetative stage is important for social evolution in nature.

Another way that high relatedness can be generated is by active processes, wherein individuals specifically take action to interact with genetic relatives (West *et al.*, 2007). This kind of identification and preferential treatment of relatives over nonrelatives is called kin discrimination (Fletcher and Michener, 1987; Strassmann, 2016; Tsutsui, 2004). Some Dictyostelids like *D. purpureum* have strong kin discrimination and sorting mechanisms (Mehdiabadi *et al.*, 2006; Mehdiabadi *et al.*, 2009; Sathe *et al.*, 2014). In this species different genotypes aggregate together, then sort into two different slugs that go on to make their own mostly clonal fruiting bodies (Fig 1.4). *D. giganteum* may also have strong kin discrimination mechanisms that vary by strain, ranging from those that aggregate little with others to those that form clonal clumps within

slugs but still fruit together, though sample sizes in these studies were very small (Kaushik *et al.*, 2006; Sathe *et al.*, 2014).

In *D. discoideum*, the degree of segregation of mixed genotypes into separate fruiting bodies varies among studies and can be quite modest (Flowers *et al.*, 2010; Gruenheit *et al.*, 2017; Ostrowski *et al.*, 2008). Gilbert et al. (2012) mixed co-occurring wild genotypes in equal proportions and found a small but significant increase in relatedness due to kin discrimination. They also found lower levels of relatedness within these chimeras compared to wild fruiting bodies and found fewer clonal fruiting bodies than expected given the frequency of clonal wild fruiting bodies. This indicates that kin discrimination does not fully explain the high relatedness levels found in fruiting bodies in nature.

1.5 Costs and benefits of associating with non-relatives

Overall, relatedness in natural fruiting bodies of *D. discoideum* is high, but *D. discoideum*'s aggregative social cycle makes it possible to manipulate relatedness in the lab and study its consequences. Genetic chimeras – aggregations comprised of cells of two or more genotypes – readily form in the laboratory (Strassmann *et al.*, 2000). Chimeras enjoy some benefits, but many associated costs. The main advantage of chimerism is the potential increase in the size of the aggregate (Foster *et al.*, 2002). Large slugs can move further than smaller slugs, increasing dispersal distance (Foster *et al.*, 2002). An increase in aggregate cell number could also result in taller fruiting bodies, which could increase the chance of dispersal by a passing invertebrate.

The costs of chimerism become apparent when controlling for this size advantage (Fig 1.5). Chimeric slugs move shorter distances than clonal slugs when started with the same number of cells (Foster *et al.*, 2002). This could result from competition among the genotypes to avoid the pre-stalk region located in the front of the slug and move towards the posterior pre-spore region.

A mechanism for this could be incompatibility between allotypes of the *tgr* recognition system (see below) that reduces slug movement by affecting adhesion of cells within the slug (Gruenheit *et al.*, 2017; Hirose *et al.*, 2015).

Perhaps the biggest fitness consequence to cells in a chimera is the potential for cheating or being cheated (Fig 1.1). In chimeras, the benefits of the social cycle may not distribute equally between all of the genotypes involved. Some genotypes could contribute less towards stalk production and make more spores. We define this as cheating for *D. discoideum*. For example, if cells of genotypes A and B form a chimeric aggregate at a 50:50 ratio then, in the absence of cheating, half the spores in the resulting fruiting body should belong to genotype A and half to B (Fig 1.6A). However, if A cheats B, we may find that 60% of the spores are genotype A while only 40% are genotype B.

There are three forms of cheating-related spore-stalk allocation strategies: fixed, facultative, and obligate (Fig 1.6) (Buttery *et al.*, 2009; Strassmann and Queller, 2011) . Fixed cheating occurs when cells of one genotype inherently invest more into spore production and less into stalk production than cells of another genotype (Fig 1.6B). On their own the two genotypes will differ in fruiting body morphology. When these two genotypes form a chimera, one genotype will be overrepresented in the spores even though it is not acting any differently than it would on its own. Variation in clonal allocation could be a result of natural selection on other traits favoring different optimal spore-stalk allocation, but it could also have evolved for the purpose of cheating advantage when in chimeras.

In contrast, facultative cheaters change their behavior in response to the presence of another genotype, and can be further partitioned into self-promoting and coercive cheaters (Fig 1.6C)

(Buttery *et al.*, 2009). Self-promotion occurs when a genotype selfishly increases its spore investment in a chimera. Coercion occurs when the partner genotype is coerced to increase its stalk investment in a chimera. For example, consider genotypes A and B with the same clonal spore-stalk allocation of 80:20. If A cheats through self-promotion then A's allocation could change to 90:10 in chimeras, whereas if A cheats through coercion then it could force B's allocation to change to 70:30.

Obligate cheaters, or social parasites, are another form of cheater that cannot develop properly on their own and must have a victim to exploit (Fig 1.6D). These cheaters threaten multicellularity itself because if they grow and spread they could eventually eliminate those able to form stalks, leading to their extinction since *D. discoideum* can only make hardy spores with the formation of stalked fruiting bodies. Obligate cheaters evolve readily in the lab (Ennis and Sussman, 1975; Kuzdzal-Fick *et al.*, 2011; Santorelli *et al.*, 2008) but they are likely rare or nonexistent in nature because they have not been found in *D. discoideum* despite the screening of thousands of natural isolates (Gilbert *et al.*, 2007).

Cheating is common in chimeras; one genotype often dominates the other genotype and produces more spores (Strassmann *et al.*, 2000). There is evidence for both fixed and facultative strategies in wild clones (Buttery *et al.*, 2009). Variation in fixed cheating strategies partially explains the linear hierarchy of exploitation by genotypes (Buttery *et al.*, 2009; Fortunato *et al.*, 2003), but the exact extent of exploitation by a genotype also depends upon its competing partner genotype in the chimera, consistent with some occurrence of facultative cheating (Buttery *et al.*, 2009).

There are two other kinds of evidence consistent with facultative cheating. First, since there is less benefit to one's own spores by investing in stalk in chimeras, facultative cheating via self-

promotion would predict that chimeras should produce more spores. There is evidence for an increase in overall spore production in chimeras (Buttery *et al.*, 2009) but the evidence is ambiguous about whether this results in shorter stalks. Some studies show that chimerism had no significant effect on fruiting body morphology, implying there was no reduction in stalk height (Foster *et al.*, 2002) or showed no consistent pattern in change in stalk height (Votaw and Ostrowski, 2017), whereas another study reports that chimerism results in significant change in fruiting body architecture (Buttery *et al.*, 2009). These inconsistencies might be because stalk height is much harder to measure and more variable than spore investment. Clearly more work is needed on this important topic.

Second, there is some evidence that cheating is frequency dependent (Madgwick *et al.*, 2018), such that the rarer the genotype is within a chimera, the more it cheats. This provides more evidence for facultative cheating, where spore-stalk investment is modulated on the basis of relatedness to the group. It makes sense adaptively because a rare genotype that makes stalk cells will mostly be benefiting the other genotype (Madgwick *et al.*, 2018). However, another study reports no or weak frequency dependence depending on the genotypes examined (Buttery *et al.*, 2009).

1.6 Genes for cheating

D. discoideum's rich history as a model system allows one to identify genes that control cheating behavior. Restriction enzyme mediated integration (REMI) is a powerful tool for gene identification, wherein gene knock-outs are created by inserting DNA fragments into the genome. Ennis et al. (2000) generated a large pool of REMI mutants that were each randomly disrupted for a single gene function, then selected for preferential spore production. They identified a mutant called *chtA* (a *fbxA* knockout) that is an obligate social cheater, which is able

to cheat in chimeras but is developmentally deficient and produces few spores when grown clonally.

Using a similar approach, Santorelli et al. (2008) subjected pools of REMI mutants through several cycles of spore production, but obligate cheaters were excluded by only considering clones capable of normal fruiting body development when clonal. This resulted in the identification of 167 candidate cheater genes that increased in frequency. On characterizing a smaller subset of 31 confirmed cheater genes, they found that 45% of these genes were not significantly different from wild type in their sporulation efficiency when grown clonally. These mutants are facultative cheaters that are able to produce more than their share of spores when in chimera but cooperate normally when clonal.

One such facultative cheater is *chtB* (Santorelli *et al.*, 2013). A *chtB* mutant is able to form a normal fruiting body when alone, but upon mixing in equal proportion with a wild genotype, *chtB* mutants contribute nearly 60% of the spores. This mutant shows no trade-offs in general morphology, spore production, or germination efficiency. Similarly, *chtC* mutants are also facultative cheaters that cheat by affecting pre-stalk differentiation and show no trade-off with general morphology or spore production (Khare and Shaulsky, 2010). Determining the reasons why mutations that disrupt such gene functions have not spread in wild populations despite an apparently cost-free cheating strategy is an interesting avenue of future research.

1.7 Power

Each cheater gene must use some particular mechanism to ensure that it gets into spores. The number and functional diversity of such genes suggests that there are many such levers of power (Santorelli *et al.*, 2008). But there are also some general environmental factors that affect the

power to win in chimeras and, if cheating is important in nature, amoebas will likely have evolved to exploit these levers of power as well.

In animals, contests and fights are often won by the largest individuals or those in the best condition. Consistent with this, *D. discoideum* cells fed glucose are more likely to become spores over those starved of glucose (Castillo *et al.*, 2011; Leach *et al.*, 1973; Thompson and Kay, 2000). Similarly, cells weakened with acid are less likely to become spores (Fig 1.7) (Castillo *et al.*, 2011). There is a possibly related effect of stage of the cell cycle, where cells in the period shortly after cell division are more likely to become stalk than those that have had more growth and command more resources (Fig 1.7) (Araki *et al.*, 1994; Azhar *et al.*, 2002; Gomer and Firtel, 1987; Gruenheit *et al*., 2018).

However, the first cells to starve, which should have fewer stored nutrients, tend to become spores (Kuzdzal-Fick *et al.*, 2010). One explanation is that although resource-rich cells have an advantage, this could be overcome by cells that have time to prepare their "weapons" and become superior competitors (Castillo *et al.*, 2011; Queller and Strassmann, 2018; Strassmann and Queller, 2011). It is interesting from an evolutionary perspective that amoebae would join a signaler of starvation that is itself taking the selfish role and expecting the later joiners to become the altruistic stalks.

These results are consistent with cells using whatever environmental advantage they can to be among the 75-80% to become spores. It has been argued that competition is a sufficient explanation of the altruistic behavior in *D. discoideum*, that it is a pure game of power and individual selection, rather than kin selection to help relatives (Atzmony *et al.*, 1997). In this view, all cells try to become spores and the losers are forced to form stalk.

However, kin selection and individual selection make different predictions about the relative strength of purifying selection in genes expressed in prespore and prestalk cells, with the evidence supporting kin selection (Noh *et al.*, 2018). The strength of purifying selection on a gene depends upon the fraction of individuals that express it (Van Dyken and Wade, 2010). Since only 1 in 5 cells become stalk, purifying selection against mildly deleterious mutations will be four times less effective in prestalk cells than prespore cells. Thus, individual selection predicts that prestalk genes should be at least four times more polymorphic than prespore genes. Under kin selection, all selection on prestalk cells is mediated through indirect selection on the related spore cells. Accounting for the observed levels of relatedness in fruiting bodies (0.86 – 0.97 (Gilbert *et al.*, 2007)), the levels of purifying selection in prestalk genes should be only 1.03 – 1.17 times as variable in the prespore genes. The observed relative strength of purifying selection is consistent with the prediction from kin selection.

Even if power could fully explain which cells lose, it cannot explain the subsequent behavior of these losers. They build a very complex stalk, and this behavior can be heritable only via related spores because stalk cells do not reproduce. Kin selection and power can of course operate together, with power accounting at least partly for which cells become stalk cells but kin selection explaining their ability to stop competing and act instead to contribute to spore success.

1.8 Cooperation is maintained by control of cheaters

In *D. discoideum*, the altruistic behavior of some cells can be exploited by cheater mutants. Though exploitation is unlikely to happen if relatedness is high and cheaters are forced to primarily interact with themselves*, D. discoideum* nonetheless has mechanisms that can control cheating and prevent cheaters from taking over a population including allorecognition, pleiotropy, and evolved resistance.

1.8.1 Control of cheating by high relatedness

The very high relatedness within natural fruiting bodies should act as a strong control on cheating because different genotypes will usually be in different fruiting bodies. In an experimental demonstration of how high relatedness can control cheating, relatedness above 0.25 prevented an obligate social cheater called *chtA* from increasing in frequency when mixed with its non-cheating ancestor AX3 (Gilbert *et al.*, 2007). Similarly, low relatedness can select for cheater mutants which conversely indicates the importance of high relatedness for controlling them. When 24 initially clonal lines of *D. discoideum* were evolved at low relatedness (new clones that emerged by mutation were randomly mixed among all the others in each social generation) for 31 social cycles or about 290 cell divisions, clones in the resulting populations significantly cheated their ancestor and included many obligate cheaters (Fig 1.8) (Kuzdzal-Fick *et al.*, 2011). These examples show that low relatedness allows the spread of both facultative and obligate cheaters.

1.8.2 Control of cheating by allorecognition

Cooperation can be stabilized when cooperators direct their cooperation towards those that have a shared specific gene for cooperation and not to those that lack it, called greenbeard recognition (Fig 1.4) (Dawkins, 1976; Hamilton, 1964a; Hamilton, 1964b). This is a mechanism by which alleles directly recognize one another, different from genetic relatedness which is based on the probability that both individuals share the gene for cooperation.

One set of genes that fits these criteria are a pair of tightly linked, highly variable cell adhesion genes of *D. discoideum* called *tgrB1* and *tgrC1,* which are essential for development (Benabentos *et al.*, 2009; Gruenheit *et al.*, 2017; Hirose *et al.*, 2011; Hirose *et al.*, 2015). These

genes encode a ligand-receptor pair anchored in the cell membrane (Hirose *et al.*, 2017). As would be expected for a functioning allorecognition system, they are highly polymorphic, with the highest levels of both allelic and total sequence variation in the *D. discoideum* genome. Their sequence dissimilarity and binding affinity correlates with the degree of genotype segregation into separate fruiting bodies (Benabentos *et al.*, 2009; Gruenheit *et al.*, 2017). This strongly suggests that the *tgr* genes are responsible for allorecognition. Furthermore, all genotypes aggregate together but those with sufficiently different *tgr* genes then segregate into distinct clumps within the aggregate and then into separate slugs (Gruenheit *et al.*, 2017; Hirose *et al.*, 2011), although slugs may later fuse to form chimeric fruiting bodies (Ho and Shaulsky, 2015). Incompatible *tgr* genes can also prevent obligate social cheaters from invading because cheaters that lack the matching Tgr proteins are excluded from the final fruiting body (Ho *et al.*, 2013). Even if *tgr* genes result in incomplete sorting in fruiting bodies, earlier sorting within the aggregate may prevent cheating if cells decide whether to become spore or stalk based on their very close neighbors. Fusion at the slug stage may not lead to much cheating if, as some evidence suggests, cheaters act primarily at earlier stages (Ho and Shaulsky, 2015) and such fusion might enhance fitness through larger fruiting bodies and better dispersal. Thus, these greenbeard genes may function more to limit exploitation within fruiting bodies than to cause sorting into kin groups in the fruiting bodies.

1.8.3 Control of cheating by pleiotropy

When a gene or set of tightly linked loci encoding a cooperative behavior also has another essential function, cooperation can be maintained because cheaters (those that lack the gene) cannot survive. This is called pleiotropy, when a single gene influences multiple phenotypes. In

general, pleiotropy can hamper the evolution of a trait because selection on that trait also affects other traits.

In *D. discoideum*, several genes cause cheating when they are knocked out, but also have an essential function. For example, the obligate social cheater mutant *chtA* pays a pleiotropic cost: it cannot make spores on its own and chimeric fruiting bodies that contain more *chtA* produce fewer spores (Ennis *et al.*, 2000; Gilbert *et al.*, 2007).

Another example is the gene *dimA*, which is required to receive the signaling molecule DIF-1 that causes differentiation into prestalk cells. Absence of this gene, and thus blindness to the DIF-1 signal, should allow cells to avoid becoming stalk cells. However, cells lacking this gene are excluded from becoming spores as well by an unknown mechanism. Here, cheating on prestalk cell production yields an even greater reduction in spores so it should be selected against in nature (Foster *et al.*, 2004).

A third example of pleiotropy maintaining cooperation by preventing the evolution of cheaters are the *csA* mutants, which lack functional gp80 adhesion proteins. Cells with this mutation cheat their ancestor AX4, presumably because during the slug stage they slide to the prespore region at the back of the slug. However, these mutants can only act as a cheater when grown on agar, but not the more realistic substrate of soil (Queller *et al.*, 2003).

1.8.4 Control of cheating by evolved resistance

Cooperators can evolve to resist cheaters without evolving to become cheaters themselves, even when cheaters can evolve in response (Hollis, 2012; Khare *et al.*, 2009; Levin *et al.*, 2015). Khare et al. (2009) found that introducing a cheater into a randomly mutated population of *D. discoideum* selected for mutants that resisted cheating but did not cheat the ancestral strain or the

original cheater. In addition, Hollis (2012) mixed two genotypes, one which strongly cheated the other, and found that the non-cheater evolved resistance to cheating. These studies show that cooperators can evolve to resist cheaters without cheating them in turn.

Levin et al. (2015) tested if evolved obligate social cheaters cheated on their contemporaries in addition to their ancestors. They found that the contemporaries resisted the cheaters without themselves cheating. This shows that resistors can evolve in populations where obligate cheaters had already evolved (Fig 1.8), but before the cheaters have swept through the population. This indicates that the evolution of resistance to cheating can be quite rapid.

Evolved resistance to cheating could in turn select for stronger cheating in a positive feedback loop, called an arms race or red queen dynamics (Queller and Strassmann, 2018). This would be similar to the dynamics between hosts and pathogens, where pathogens continually evolve to better infect their hosts while their hosts evolve in response to resist the pathogens.

1.9 Relevance of cooperation and cheating for *D. discoideum*

Lab studies on *D. discoideum* have advanced our knowledge about many aspects of cheating behavior, such as its genetic basis and the various mechanisms that allow for its control. However, the relevance of this behavior in nature has been questioned. Since we cannot observe these behaviors in the wild, we may overinterpret such responses in the lab.

Apparent cheating could be a result of trade-offs associated with other life-stages. Hence, what appears to be an outcome of social interaction could be due instead to selection on other non-social traits (Tarnita, 2017). One study suggests that unequal spore numbers in the fruiting body may not translate into unequal social success because spore production trades off with spore viability (Wolf *et al.*, 2015). Natural variation between genotypes in spore production is

negatively correlated with their spore size, which in turn is correlated with spore viability. Genotypes that produce more spores in chimeras may sometimes do so by producing smaller, less viable spores, and hence gain no cheating advantage. However, another study found no correlation between spore production and spore viability when averaged by genotype (Votaw and Ostrowski, 2017).

Some studies argue that there is a trade-off between staying a vegetative cell and becoming a spore (Dubravcic *et al.*, 2014; Tarnita *et al.*, 2015). "Loner" amoebas that do not join the aggregate remain viable and benefit from a head start over cells that have become spores and thus need time to germinate into vegetative cells (or alternatively have been dispersed away). Thus genotypes that appear to be victims of cheating because they produce fewer spores in chimeric fruiting bodies could instead simply be strains that produce more loner cells. However, nothing is known about the frequencies and viabilities of loner cells in nature to test this proposed trade-off.

Importantly, insights from population genomics and molecular evolution suggest that cheating and conflict in chimeras are not just laboratory artifacts. Conflict can be an exceptionally strong and persistent selective pressure driving evolutionary arms races (Dawkins and Krebs, 1979; Queller and Strassmann, 2018; Van Valen, 1973). If cheating occurs in nature for *D. discoideum*, then it may cause resistance to cheating to evolve, as has been observed in the lab (Hollis, 2012; Khare *et al.*, 2009; Levin *et al.*, 2015). This could lead to an escalating arms race in which new cheating genes and new resistance genes sweep through the population. This in turn would lead to increased adaptive divergence for the genes involved. Another possibility is that there is negative-frequency dependence to cheating as has also been observed in a laboratory setting (Madgwick *et al.*, 2018). This means that cheaters prosper only when they are in low numbers.

This would lead to increased non-synonymous variation within species and decreased non-synonymous divergence between species for the genes involved. However, if cheating behaviors are not important and do not experience strong adaptive selection, then their patterns of sequence variation should be similar to other genes in the genome, influenced primarily by drift and purifying selection.

Ostrowski et al. (2015) analyzed variation between and within species sequence in 160 candidate cheater/cooperation genes identified from the Santorelli et al. (2008) REMI mutant study. The signatures in sequence variation were most consistent with greater-than-normal negative-frequency dependent selection, acting to maintain both cheaters and cooperators as a balanced polymorphism (Ostrowski *et al.*, 2015). This finding is consistent with the laboratory finding that cheating is frequency dependent (Madgwick *et al.*, 2018).

Noh et al. (2018) used RNA-seq to identify a second set of cooperation/cheater genes by screening for genes that change expression in chimeric mixtures of two genotypes. It is in this exact context that cheating is likely to be adaptive, and hence if any genes function specifically in cheating or resistance to cheating, these are excellent candidates. They identified 79 genes that significantly differed in their expression in chimeras compared to controls. These genes show elevated rates of adaptive evolution α compared to the genomic background. This is consistent with escalating arms race conflict leading to high rates of adaptive evolution in these genes.

It is not clear why one set of genes showed excess balancing selection and the other showed excess adaptive fixations. That said, the gene sets are quite different; the first study used REMI mutants selected for cheating while the second set used naturally expressed genes that may include resistance genes. In any case, both these studies provide strong evidence for the historical

importance of cheating in the wild. Several other studies strengthen this claim. First, mutation accumulation experiments show that random mutations often tend to decrease cheating ability, which is consistent with cheating being a fitness component in nature, although the effect was not strong (Hall *et al.*, 2013). Second, the presence of allorecognition systems such as the *tgr* genes indicates that avoiding non-kin that might harm or cheat is important for *D. discoideum*. Finally, there are other apparent adaptations that seem consistent with cheating in the wild: reduced slug migration in chimeras (Foster *et al.*, 2002), allocating more to spores when in chimera (Buttery *et al.*, 2009) and even more for minority genotypes in chimeras (Madgwick *et al.*, 2018). Although non-adaptive explanations could be possible, such complex responses make sense if cheating when with non-relatives and cooperating when with relatives actually conferred a fitness benefit in the wild.

1.10 Other domains of cooperation and conflict

D. discoideum's unique social cycle makes it useful for studies on the evolution of cooperation and conflict, and it also engages in cooperation and conflict in other parts of its life cycle.

1.10.1 The sexual cycle

The formation of the macrocyst in *D. discoideum's* sexual cycle involves uniquely social processes (Fig1.9). When amoebas are starving under wet, phosphorus-poor conditions, two individuals of different mating types can fuse into a diploid zygote (Bloomfield, 2013; Bonner, 1967; Kessin, 2001). The zygote emits a cAMP signal that draws other cells in the vicinity towards it. Many of the attracted peripheral cells are consumed by the zygote for nutrition, and the rest construct a cellulose wall around the aggregate before they are themselves consumed.

Following this, the zygote undergoes recombination, crossing over, and meiosis, forming many recombinant haploid cells.

The sexual cycle involves an act of altruism by the peripheral cells, as they give up their lives. It is unlikely that these cells are simply victims because they actively further the success of the zygote by building the macrocyst wall around it, but their sacrifice does potentially set the stage for social conflict over which cells are sacrificed. For example, each mating type might prefer to evade consumption to some degree and allow cells of the other mating type, its non-relatives, to provide most of the sacrifices necessary to construct the macrocyst (Douglas *et al.*, 2017). One way this could be measured is if a genotype produces a disproportionate number of macrocysts – a rare genotype should prefer more macrocysts be made because the common genotype will make up most of the food, while a common genotype should prefer fewer for the same reason. However, when one genotype is rare it usually does not cause disproportional investment in macrocysts, which instead appear to be limited by partner availability (Douglas *et al.*, 2017).

Sexual reproduction happens often in *D. discoideum* in the wild as evidenced by high recombination rates (Flowers *et al.*, 2010), but it is difficult to get the full process to occur in a laboratory setting (Kessin, 2001). Despite this, major advances have been made in recent years. The sex-determining locus is known and the presence of three different mating types has been confirmed (Bloomfield *et al.*, 2010). A recent study has revealed an interesting mode of triparental inheritance in lab crosses involving more than two gametes, where two parents contribute to the nuclear genome and the mitochondrial genome comes from the third (Bloomfield *et al.*, 2018). Much is still unknown about the sexual cycle, and it provides a rich area for future study.

1.10.2 Cooperative predation

A recent study suggests that vegetative growth in *D. discoideum* while preying on bacteria might not be asocial, but instead may involve cooperative predation (Rubin *et al.*, 2019).They found that *D. discoideum* growth is positively correlated with amoeba density, and mutants that grow poorly on live bacteria can be rescued by the presence of wild-type amoebas and synergistic mutants. They suggest this is due to the secretion of diffusible factors by wild-type cells that facilitates mutant growth, though the molecule mediating such an interaction has not yet been identified. Another study showed that *D. discoideum* plated in the presence of high densities of the bacterium *Escherichia coli* could proliferate only when plated at high densities themselves (DiSalvo *et al.*, 2014). More work in this direction could clarify the role of cooperative predation in *D. discoideum*.

1.10.3 Cooperation and conflict between species

In addition to being a valuable model organism for studying cooperation and conflict within a single species, *D. discoideum's* interactions with symbiotic bacteria can also be informative about cooperation and conflict between species. Roughly one third of wild-collected *D. discoideum* strains harbor bacterial endosymbionts belonging to the genus *Burkholderia,* with which they have a complex relationship involving both cooperation and conflict (DiSalvo *et al.*, 2015). *Burkholderia*-infected *D. discoideum* suffer some toxicity but can carry other more edible species of bacteria through their social cycle, which improves *D. discoideum*'s fitness when sorus contents are dispersed to environments without suitable food (Brock *et al.*, 2011).The extent to which *D. discoideum* and *Burkholderia spp.* are friends or enemies is likely to depend on strain-to-strain variation and environmental context. In addition, *D. discoideum* is known to

associate more transiently with a host of other bacterial taxa, including both edible and inedible strains (Brock *et al.*, 2018). Just as *D. discoideum's* management of conflict with cheaters within its own species can inform us about the benefits and constraints of multicellularity at large, the ways *D. discoideum* and *Burkholderia* interact with and evolve against this larger microbiome can model the important relationships between multicellular eukaryotes and their bacterial microbiotas in general.

1.11 Conclusion

Clearly, *D. discoideum* has provided profound insights into both the proximate (how) and ultimate (why) explanations for the evolution of cooperation and control of conflict. This has been facilitated by the fusion of two rich fields: cutting-edge molecular techniques and social evolution theory. However, there are many questions that are yet to be fully explored and resolved. Why is there a kin recognition system if it only weakly increases relatedness? How important is frequency dependence for determining cheating behavior? What conflicts occur in the sexual stage, and how do they manifest? How is cooperation between non-relatives enforced in the sexual stage? What are the relationships between *D. discoideum* and members of its microbiome, and how do these relationships evolve? Future work will address these questions and many others, as we still have much to learn from *D. discoideum*.

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1.13 Figures

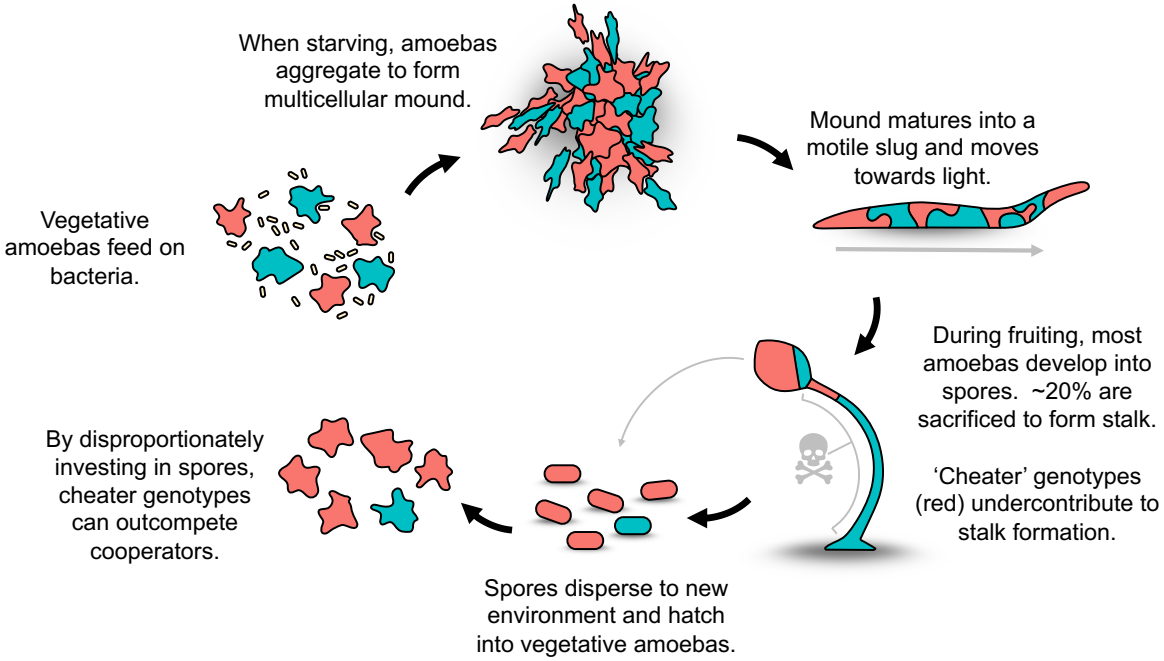

Figure 1.1. Social cycle of *D. discoideum* –When starved, single-celled amoebas aggregate into a slug-like multicellular body, then fruit. Fruiting body production requires the sacrifice of a minority of cells to produce a stalk. The potential for aggregation of multiple genotypes into a chimeric fruiting body gives opportunity to selfish 'cheater' genotypes (red), which benefit from but do not contribute to the stalks produced by other genotypes.

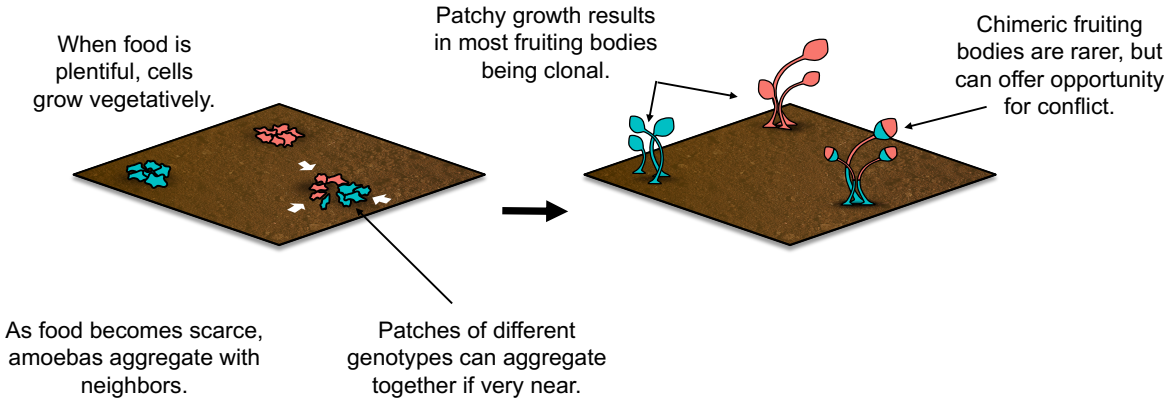

Figure 1.2. *D. discoideum* relatedness in nature – *D. discoideum* fruiting bodies collected from nature are usually clonal. Clonal fruiting bodies likely result from limited dispersal leading to a patchy distribution of genotypes, such that cells are likely only to interact with clonemates. Millimeter-scale distances between genotypes are likely sufficient to promote high relatedness. Nonetheless, a minority of wild fruiting bodies are chimeric – comprising cells derived from multiple genotypes – and presumably occur where clonal patches of different genotypes intersect.

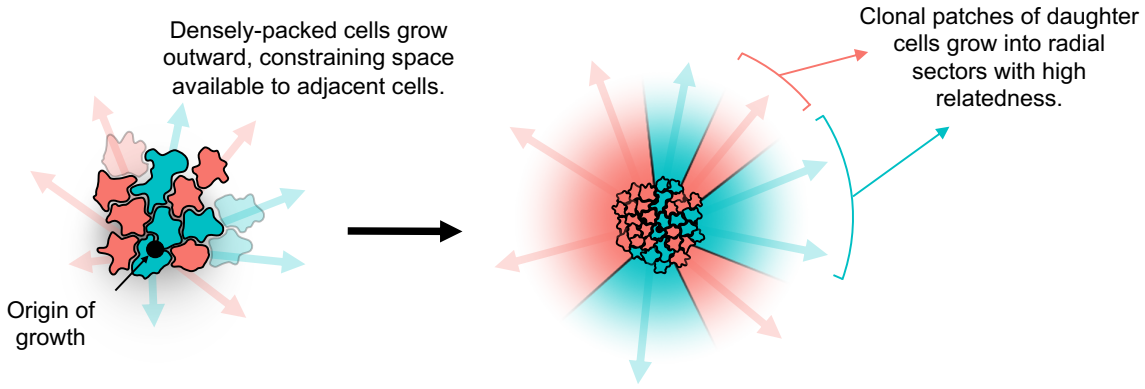

Figure 1.3. Genetic demixing in *D. discoideum* – Structured growth of an initially well-mixed (low-relatedness) population can produce patches of high relatedness due to the space constraints imposed on densely-growing cells. Cells on the periphery expand outward into radial sectors of clonal daughter cells. Whether genetic demixing can occur in natural soil is unknown.

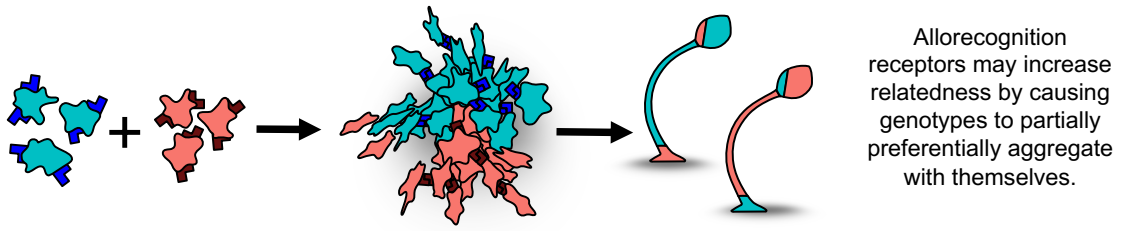

Figure 1.4. Allorecognition in Dictyostelids – There is evidence for varying degrees of self-sorting among Dictyostelid species. Cells bearing the same alloreceptors preferentially bind to one another in an aggregate and thus may increase relatedness (and thereby reduce the opportunity for cheating) within developing fruiting bodies by sorting kin into distinct regions of the aggregate.

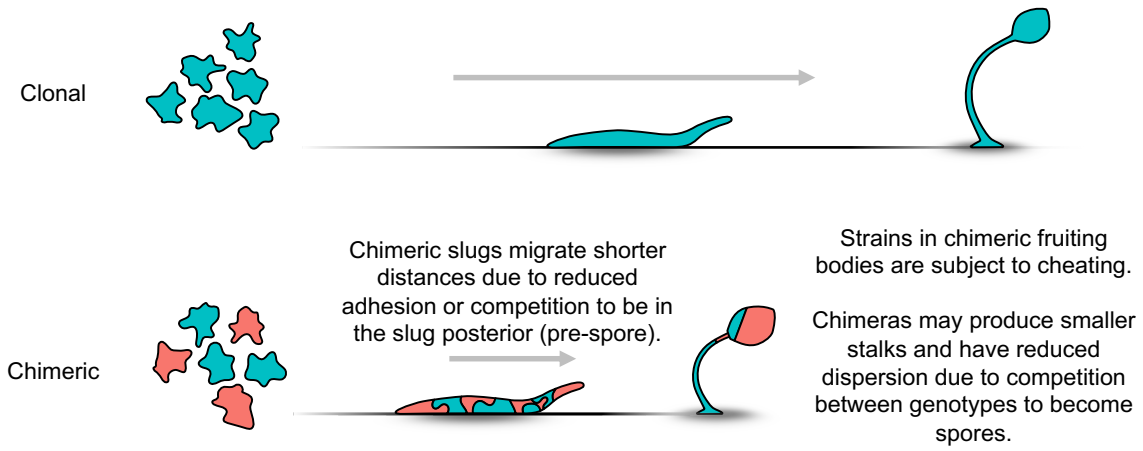

Figure 1.5. Costs of chimerism in *D. discoideum* – While forming a chimera may sometimes be beneficial if it results in a larger aggregate, chimeric slugs are less motile than clonal slugs of the same size, which may result from conflict between genotypes within the slug. Chimeric fruiting bodies also are subjected to the risk of being cheated upon, and may produce smaller stalks than clonal fruiting bodies.

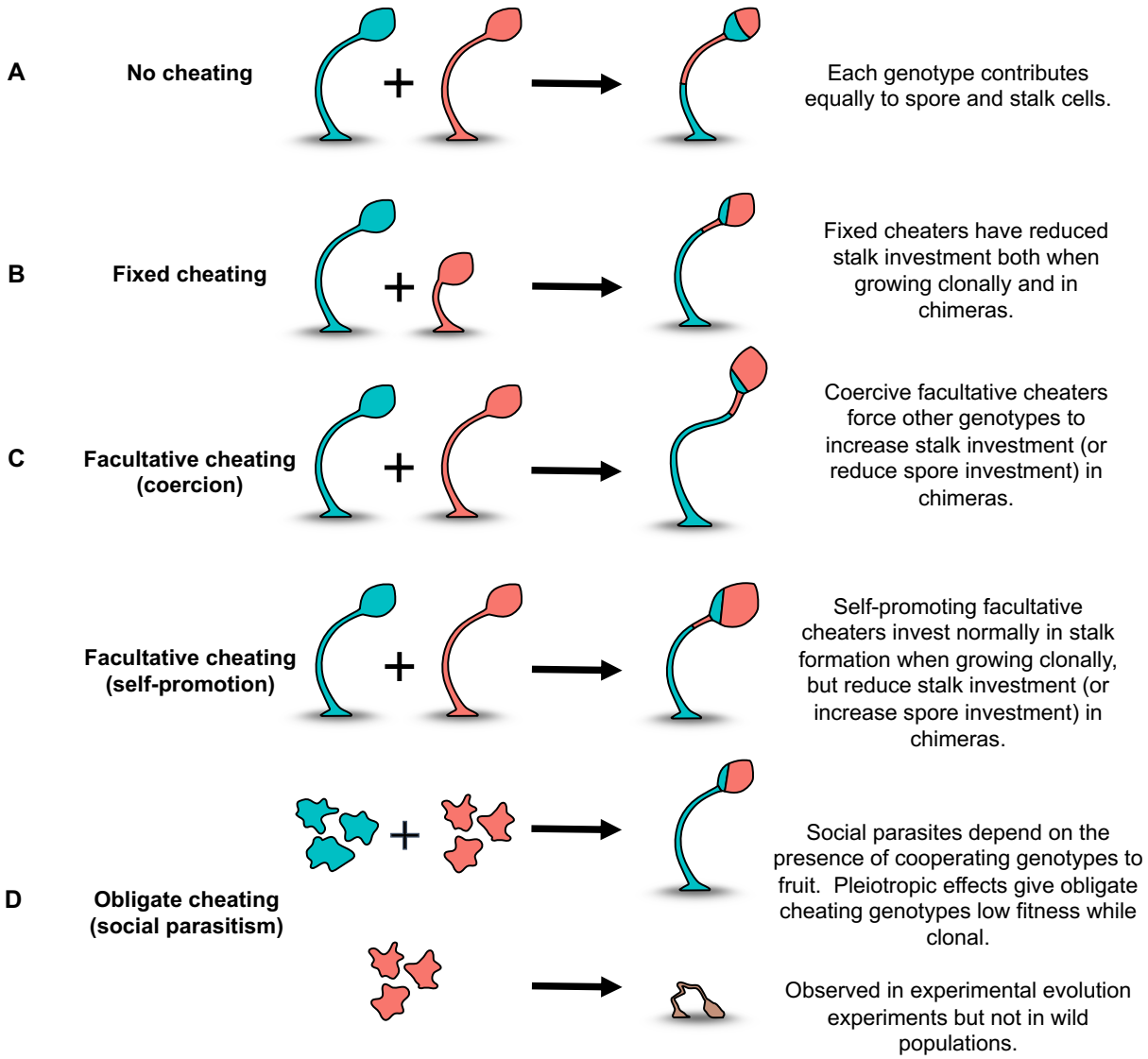

Figure 1.6. Cheating strategies in *D. discoideum* – **A)** In the absence of cheating, two genotypes that aggregate together in a 50:50 ratio will each contribute half of the resulting fruiting body's stalk and half of its sorus. **B)** Fixed cheaters produce a higher spore:stalk ratio when grown clonally or in a chimera. These strategies may or may not have evolved due to the social benefits of cheating in chimeras. **C)** Facultative cheaters take advantage of other genotypes in a chimera by either forcing other genotypes to reduce their spore:stalk ratio

(coercion) or increasing their own spore:stalk ratio (self-promotion). **D)** Obligate cheaters depend on the presence of other genotypes to fruit. They have been observed in experimentally evolved populations in the laboratory never isolated in nature.

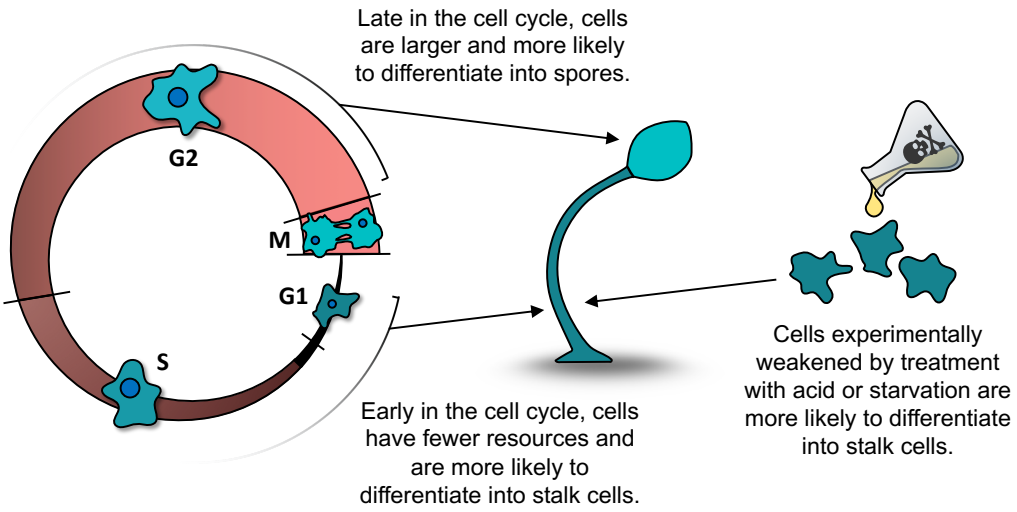

Figure 1.7. Power impacts cell fate in *D. discoideum* – Power partially determines the fate of cells within a developing fruiting body, such that more powerful cells are more likely to become spores while less powerful cells are forced to become stalk cells. Cells experimentally weakened with acid or starvation are more likely to become stalk cells. Cells late in the cell cycle are larger and have more resources than cells that have recently divided, and thus tend to differentiate into spores upon entering the social cycle.

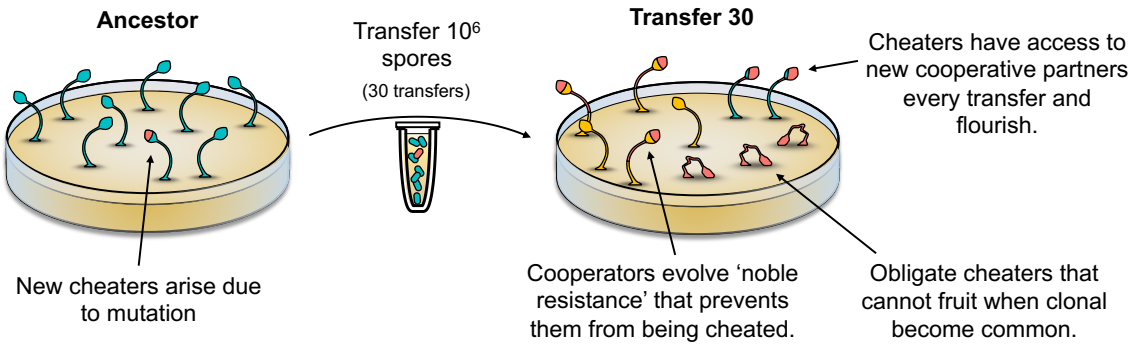

Figure 1.8. Relatedness affects evolution of cheaters in *D. discoideum* – Kuzdzal-Fick et al experimentally evolved replicate lines of *D. discoideum* under treatments enforcing low relatedness. Transfers were performed using 10^6 spores gathered from across the plate, effectively mixing the population each transfer. Cheater mutants were repeatedly exposed to new partners to exploit and so prospered. Obligate cheaters incapable of fruiting on their own readily evolved. Eventually, the pressure exerted by cheaters caused other genotypes to evolve 'noble resistance' – these strains could resist cheating without being cheaters themselves.

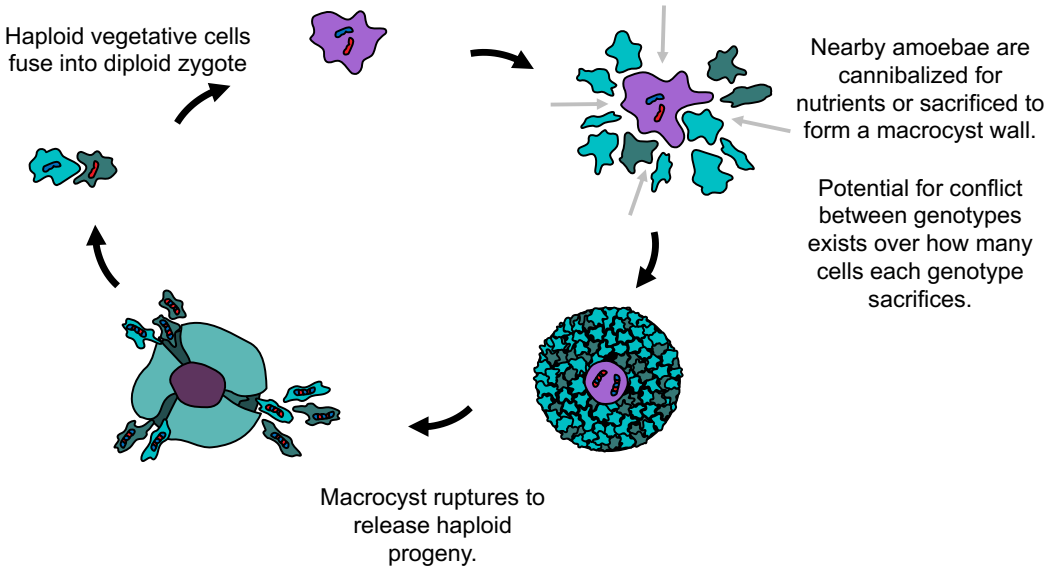

Figure 1.9. The sexual cycle of *D. discoideum* *–D. discoideum* undergoes a sexual cycle in nature wherein haploid amoebas of different mating types fuse into a diploid zygote and induce nearby cells to sacrifice themselves to provide nutrients and to produce a macrocyst wall. This process may drive conflict between genotypes over which cells are sacrificed.

Chapter 2: Limits to the Spread of an Obligate Social Cheater in *Dictyostelium discoideum*

James Medina, David Queller, Joan Strassmann

2.1 Abstract

Cooperation is widespread across life, but its existence can be threatened by exploitation. Social cheaters can be obligate, incapable of contributing to a necessary function, so spread of the cheater leads to loss of the function. In the social amoeba *Dictyostelium discoideum*, obligate social cheaters cannot become dead stalk cells that lift spores up for dispersal, but instead depend on forming chimeras with fully functional altruistic individuals for forming a stalk. Obligate cheaters in *D. discoideum* are known to pay the cost of being unable to form fruiting bodies on their own. In this study we discovered that there are two additional costs that can apply to obligate cheaters. Even when there are wild-type cells to parasitize, the chimeric fruiting bodies that result have shorter stalks that are disadvantaged in dispersal. Furthermore, we found that obligate cheaters were overrepresented among spore cells in chimeras only when they were at low frequencies. Failure to develop into viable fruiting bodies on their own, negative frequency-dependent cheating, and shorter fruiting bodies represent three limits on obligate social cheating so it is not surprising that they have not been found in nature.

2.2 Introduction

Cooperative behavior is common in nature, but cooperators are vulnerable to cheaters who can gain the benefits of cooperation without paying the costs (Bourke 2011). In order for cooperation to persist, strong conflict between cooperators and cheaters must be mitigated (Smith and Szathmary 1997; Michod 2000; Queller and Strassmann 2018). Cheating can be reduced or

eliminated by natural selection if the benefits of cooperation preferentially go to relatives because relatives are likely to share the gene or genes underlying the cooperative behavior. This is called kin selection, and is based on inclusive fitness theory, because individuals maximize their inclusive fitness, which includes their personal fitness as well as their effects on the fitness of their relatives, modified by how closely related they are (Hamilton 1964a,b; Frank 1998; Grafen 2006).

The social amoeba *Dictyostelium discoideum* can form a multicellular fruiting body but requires altruistic action by a subset of cells in order to do so. In the wild, individual amoebas live in soil and leaf litter where they prey upon bacteria. When starved, they aggregate, develop into a multicellular slug, and migrate to a new location where they form a fruiting body. In this multicellular structure a minority of cells in the slug altruistically sacrifice their lives to form a dead stalk which lifts the other cells a few millimeters above the soil as viable, hardy spores in a structure called the sorus (Kessin 2001). Altruism like this can evolve by kin selection if the spores are genetic relatives of stalk cells, provided the benefits of making a stalk are high enough.

Given that it is costly, why become stalk at all? The benefit of making a fruiting body seems to be dispersal, as those who have their stalks experimentally destroyed are dispersed less by a model insect vector *Drosophila melanogaster* than those with intact stalks (smith et al. 2014).

Aggregations can form between unrelated genotypes (Strassmann et al. 2000) or even different species (Jack et al. 2008), setting the stage for social conflict between who becomes spore and who becomes stalk. In heterogeneous aggregations, called chimeras, natural selection should favor genotypes that preferentially become spores and place the burden of stalk-building on the

other genotype. Conflict can be controlled in this system by high relatedness (Gilbert et al. 2007; Kuzdzal-Fick et al. 2011b; Inglis et al. 2017), as well as by other mechanisms such as pleiotropy (Foster et al. 2004) and a lottery-like role assignment system based on the cell cycle and nutrition (Strassmann and Queller 2011). In *D. discoideum*, the high relatedness necessary for preserving cooperation can be generated by active processes like kin discrimination (Ostrowski et al. 2008; Benabentos et al. 2009; Gilbert et al. 2012; Strassmann 2016), or by passive processes like spatial population growth and fine-scale population structure (Buttery et al. 2012; smith et al. 2016).

There are multiple ways for an amoeba to cheat (Travisano and Velicer 2004; Santorelli et al. 2008; Buttery et al. 2009; Strassmann and Queller 2011; Medina et al. 2019). Facultative cheaters will overrepresent themselves in the spores when in chimera but can still make fruiting bodies on their own. In *D. discoideum* the mutants *ChtB*$^{-}$ and *ChtC*$^{-}$ are examples of this strategy (Khare and Shaulsky 2010; Santorelli et al. 2013b) . Fixed cheaters always allocate the same amount to spores and can be overrepresented in the spores if their fixed strategy happens to be to give more to spores than their social partner does. Allocation to spore vs stalk varies in nature so this may be common (Votaw and Ostrowski 2017). Social parasites, or obligate social cheaters, cannot make fruiting bodies on their own and tend to become spore in chimera such as the mutant *ChtA*$^{-}$, better known as *fbxA*$^{-}$ (Ennis et al. 2000). Unlike the other categories of cheating, the spread of obligate social cheaters can threaten cooperation itself.

Relatedness in *D. discoideum* is high in nature which keeps obligate social cheaters such as *fbxA*- from spreading because they lack other genotypes to exploit (Gilbert et al. 2007). Consistent with this idea, no obligate social cheaters have been isolated from nature despite extensive sampling (Gilbert et al. 2007; Votaw and Ostrowski 2017). In contrast, when relatedness is experimentally

lowered in the lab, obligate social cheaters evolve readily and repeatedly (Ennis et al. 2000; Kuzdzal-Fick et al. 2011a; Inglis et al. 2017).

Aside from low fitness when alone, there may be other potential limits to the spread of obligate social cheaters. One of these is negative frequency-dependent cheating. This occurs when cheaters are overrepresented in the spores only when they are at low frequencies. When cheating is negatively frequency-dependent, obligate social cheaters do not threaten cooperation itself because they are self-limiting. There is evidence for negative frequency-dependent cheating in *D. discoideum* for some facultative cheaters (Buttery et al. 2009; Madgwick et al. 2018). We know little about frequency-dependent cheating in obligate social cheaters. The only well-studied obligate cheater, *fbxA*-, overrepresents itself in the spores at all frequencies (Gilbert et al. 2007).

Another factor that could limit the spread of obligate social cheaters is if a cheater places the entire burden of stalk production on its social partner. If the partner does not compensate by allocating more to stalk than it would in a clonal fruiting body, then the fruiting body would be shorter. This may in turn reduce the likelihood that individuals in that sorus are dispersed as often or as far. The impact of social behavior on dispersal potential via the height of fruiting bodies, rather than their presence or absence, has not yet been experimentally tested.

Here we examine the effect of social conflict between a wild clone of *D. discoideum*, NC28.1, and an obligate, non-fruiting social cheater previously evolved in the laboratory from the same clone under conditions of low relatedness, called EC2 (Inglis et al. 2017). This obligate social cheater cannot develop properly on its own, similarly to *fbxA*-, and overrepresents itself in the spores when at a 10% initial frequency relative to NC28.1. We mixed these two clones at different frequencies and measured the heights of fruiting bodies they produced and the

frequency of the obligate cheater in those fruiting bodies. We expected that fruiting bodies containing more obligate social cheaters would be shorter because the cheaters do not contribute to the stalk. Alternatively, the initial frequency of cheaters could have no effect on stalk height if the wild-type facultatively increases its allocation to stalk in order to increase the likelihood that it is dispersed, or the cheater coerces the wild-type to allocate to stalk, as has been found in some cheater mutants (Buttery et al. 2009; Santorelli et al. 2013a). As far as frequency dependence goes, we predicted that another limitation to the success of obligate cheaters would be their decline in cheating as their proportion increases.

2.3 Methods

2.3.1 Strains and culture conditions

To prepare food bacteria for *D. discoideum* clones to prey upon, we first spread non-pathogenic *K. pneumoniae* KpGe (Dicty Stock Center, dictybase.org) frozen in 80% KK2 [2.25 g KH_2PO_4 (Sigma-Aldrich) and 0.67 g K_2HPO_4 (Fisher Scientific) per liter] and 20% glycerol on an SM/5 agar media [2 g glucose (Fisher Scientific), 2 g yeast extract (Oxoid), 0.2 g $MgCl_2$ (Fisher Scientific), 1.9 g $KHPO_4$ (Sigma-Aldrich), 1 g K_2HPO_5 (Fisher Scientific), and 15 g agar (Fisher Scientific) per liter] and allowed the bacteria to grow at room temperature until single colonies appeared, which happened in about two days. We picked a single colony from this plate with a sterile loop, spread it on a new SM/5 plate, and allowed the bacteria to grow for two days in order to reach high abundance. We collected these bacteria into KK2 with a sterile loop and diluted them to 1.5 OD_{600} in KK2 (~5 x 10^8 cells, measured with an Eppendorf BioPhotometer). We used these bacteria as food for amoebas in our experiment and repeated this process anew for each of the three replicate experiments.

To grow NC28.1, the wild-type ancestor clone, from freezer stocks for use in our experiments we added spores frozen in 80% KK2 and 20% glycerol to 200µl of 1.5 OD_{600} *K. pneumoniae* suspension. We spread the mix of spores and bacteria on SM/5 plates with a sterile glass spreader, then incubated the plates at room temperature for 7 days under constant overhead light until the social cycle was complete and fruiting bodies had formed. We repeated this process for each of the three replicate experiments.

To grow EC2, the RFP-labelled obligate social cheater (Inglis et al. 2017), from freezer stocks for use in our experiments, we added amoebas frozen in HL5 (5 g proteose peptone, 5 g thiotone E peptone, 10 g glucose, 5 g yeast extract, 0.35 g Na2HPO4 * 7H2O, 0.35 g KH2PO4 per liter) with 10% DMSO to 200µl of 1.5 OD_{600} *K. pneumoniae* suspension. We used amoebas rather than spores because EC2 produces few spores on its own. We spread the mix of amoebas and bacteria on an SM/5 plate with a sterile glass spreader, then incubated the plate at room temperature for 24-48 hours until starving EC2 amoebas began aggregating. We then used a sterile loop to transfer a sample to a new plate containing fresh *K. pneumoniae* for them to prey upon. These were allowed to grow for 24-48 hrs until a vegetative front of amoebas had formed. We collected these amoebas with a sterile loop into ice-cold KK2 (see "Experimental procedures"). and ensured that the amoebas we used were clonal by plating 10 SM/5 plates with about 10 amoebas each, then picking a single clonal plaque originating from a single amoeba. We repeated this process for each of the three replicate experiments.

2.3.2 Experimental procedures

In order to obtain cells of both *D. discoideum* clones for experimental mixing, we plated amoebas (EC2) or spores (NC28.1) previously grown from freezer stocks as described above on separate SM/5 agar plates with 200µl of 1.5 OD_{600} *K. pneumoniae* suspension. We allowed the

D. discoideum spores to proliferate until they reached similar numbers to the amoebas, but before they aggregated and attempted to fruit, 24-48 hours.

We collected amoebas to make the mixtures by pouring ice-cold KK2 onto the plates, rubbing them into suspension with a gloved fingertip, then collecting and centrifuging the mixture at 10°C for 3 minutes at 1300 rpm in order to pellet the amoebas and leave *K. pneumoniae* in solution. We decanted the pellets, resuspended them in KK2, and measured their density with a hemacytometer before making the mixtures. For each treatment, we mixed 200µl of fresh *K. pneumoniae* suspension with a total of $2x10^5$ amoebas then spread the solution evenly with an ethanol-sterilized glass spreader on an SM/5 agar plate. We made mixtures of EC2 and NC28.1 with various initial frequencies of EC2 (0.0, 0.1, 0.3, 0.5, 0.7, 0.9, and 1.0) in order to generate variation in their final frequencies in fruiting bodies. We repeated this experiment three times, each on a separate day.

We collected fruiting bodies after one week at room temperature under constant overhead light to allow fruiting bodies to fully develop. On each plate, we selected three fruiting bodies at random. To do this, we placed a plate of fruiting bodies over a grid of 1cm by 1cm squares with some of the squares colored in at random. We selected three of the colored-in squares at random using a random number generator and marked each plate at the centers of each of the squares. We then individually collected the closest fruiting body to each mark with fine tweezers. For each, we pressed the sorus, which contains the spores, against the side of a 100µl tube containing 100µl of KK2, to dislodge the spores then laid the stalk on a glass microscope slide. The contents of the tube were vortexed and immediately run through an Accuri C6 flow cytometer before they stuck to the sides of plastic tubes. We used 50µl of suspended spores and recorded the numbers of

fluorescent (EC2) and non-fluorescent (NC28.1) spores. After three fruiting bodies were collected from a single plate, the stalks were covered with a cover slip and sealed with nail polish for later imaging. Stalk length was individually recorded by imaging picked stalks under a Leica S8AP0 dissecting microscope with Leica application suite software v4.1 using the "draw line" tool.

2.3.2 Analysis

We excluded several data points from the analysis for which we could not accurately measure stalk height due to damage during collection. We also excluded a data point for which very few spores were counted by the flow cytometer (<300) because the cheater proportion calculated for this sorus is probably inaccurate. We treated failed aggregates which did not fully differentiate into fruiting bodies as having a stalk height of zero. We reanalyzed these data excluding failed aggregates and found similar results.

We had a treatment consisting entirely of cheaters (EC2). Though they generally do not produce fruiting bodies, they do succeed in making a very few. We did not include those data because our main question is whether mixtures result in shorter stalks. However, we did collect spores from these fruiting bodies in order to measure the proportion of EC2 cells which retained the RFP label through the process of fruiting. We use this to correct our proportion of fluorescent cells data from the other treatments to reflect the true proportion of EC2.

In order to test our whether increasing cheater frequency yields shorter fruiting bodies, we used a linear mixed-effects model with the function lme in the nlme package in R (version 4.2.1, The R Foundation for Statistical Computing) with stalk height as the response variable, the initial cheater frequency as a fixed effect, the total number of spores per sorus as a fixed effect, and the

day of the experiment as a random effect (stalk height ~ initial cheater frequency + total spores + 1|day). We included the total number of spores as a fixed effect in our initial model because fruiting bodies may be tall simply because they develop from larger populations.

We then excluded the random effect of day by using the lm function in base R and compared the two models with the anova function in base R (stalk height ~ initial cheater frequency + total spores). We found that the two models were not significantly different ($p = 0.27$, with day: AIC = 105.67, without day: AIC = 104.89), so we proceeded with the simpler model without the effect of day. We then further simplified the model by removing the effect of total number of spores because it did not significantly affect stalk height ($p = 0.20$) (stalk height ~ initial cheater frequency).

We also tested if the obligate social cheater clone, EC2, overrepresented itself in the spores of fruiting bodies relative to its initial frequency with a linear mixed-effects model using the function lme in the nlme package in R with final cheater proportion as the response variable, the initial cheater frequency as a fixed effect, and the day of the experiment as a random effect (final cheater proportion ~ initial cheater proportion + 1|day). We excluded the random effect of day by using the lm function in base R and compared the two models with the anova function in base R (final cheater proportion ~ initial cheater proportion). We found that the two models were not significantly different ($p = 1$, with day: AIC = -47.65, without day: AIC = -49.65, so we proceeded with the simpler model without the effect of day.

We further tested if the obligate social cheater clone, EC2, overrepresented itself in the spores of fruiting bodies at each initial frequency. We used one sample t-tests to test if the mean of the

proportion of cheaters in fruiting bodies was significantly different from their initial frequency using the t.test() function in base R, setting mu to the initial cheater frequency.

2.4 Results

We mixed two clones of *D. discoidium* which were a wild-type ancestor and an obligate social cheater previously experimentally evolved from that ancestor, at various frequencies relative to one another and allowed them to form fruiting bodies together. We had two goals, to see the impact of increasing proportions of the social cheater on (1) stalk height and (2) on frequency of that cheater among the spores. We found that initial cheater proportion significantly predicted stalk height (linear model, DF = 48, t = -6.17, p = 1.37e-07). The slope of this relationship was negative (-1.34, SE = 0.22), indicating that increasing the initial cheater proportion decreased stalk height (Figure 2.1). We then reanalyzed the data excluding failed aggregates instead of treating them as having a stalk height of zero. We found that initial cheater proportion still significantly predicted stalk height (linear model, DF = 43, t = -4.83, p = 1.75e-05). The slope of this relationship remained negative (-1.22, SE = 0.25).

For the second goal on obligate social cheater representation in the spores relative to initial frequency, we found that initial cheater proportion significantly predicted final cheater proportion (linear model, DF = 38, t = 13.80, p = 2.23e-16). The slope of this relationship was positive and, most importantly for our question, significantly lower than 1 (0.85, SE = 0.06, p = 0.02), indicating the clone that did not make fruiting bodies on its own cheated its wildtype partner less as the frequency of the cheater increased (Figure 2.2).

When we tested each initial cheater frequency separately we found that cheating was evident only at lower frequencies of the cheating clone. At 0.1 initial frequency the final frequency was marginally greater than initial frequency (t = 2.1167, df = 7, p-value = 0.07207). At 0.3 initial

frequency the final frequency was significantly greater than the initial frequency (t = 3.1008, df = 7, p-value = 0.0173). At 0.5, 0.7 and 0.9 initial frequencies the final frequency was not significantly different from the initial frequency (t = 1.3555, df = 8, p-value = 0.2123; t = 1.3493, df = 6, p-value = 0.2259; t = -1.3388, df = 8, p-value = 0.2174 respectively).

2.5 Discussion

Social behavior like cooperation is common in microbes but populations of cooperators can be invaded by cheaters that benefit from cooperation without paying the cost (West et al. 2007; Ghoul et al. 2014). Obligate social cheaters in particular can pose a threat to cooperation itself. Previous studies in *D. discoideum* found that an obligate social cheater mutant called *fbxA*$^{-}$ is limited by poor spore production when it is without other clones to exploit (Ennis et al. 2000; Gilbert et al. 2007). We investigated a different obligate social cheater called EC2 for additional costs of this kind of social cheating.

Forming a fruiting body with a stalk appears to be important for *D. discoideum*'s fitness. Producing the fruiting body with its stalk is a complex process and is unlikely to be a side effect of some other selected trait. The importance of having a stalk is indicated by its presence across the entire ancient Dictyostelid family (though one small genus produces an acellular stalk) (Schilde et al. 2019). Finally, sacrificing some potential spores to produce a stalk can only be explained if there is some benefit. We have not documented the fitness cost of shorter stalks but we know that a stalk height of zero can reduce dispersal (smith et al. 2014) and it seems improbable that this disadvantage would not also apply also to at least very short stalks.

If the cheater is fully coercive, forcing the wildtype to make the stalk, we would expect to see full-length stalks up to about 80:20 cheater to wildtype because wildtype has enough cells to make a normal stalk. Stalks would get smaller only after that point. Instead we see that increasing the frequency of obligate social cheater cells yields shorter fruiting bodies across the entire range. This is consistent with the cheater being fully self-promoting, overrepresenting itself in the spores and/or underrepresenting itself in the stalk. Shorter stalks could prevent cheaters from reaching high frequencies in a population because fruiting bodies with more obligate cheaters in them may be less likely to be picked up by an insect vector, which is the function of having a stalk (smith et al. 2014).

In addition, we found that EC2, unlike *fbxA*$^{-}$, only overrepresented themselves in the spores when they were mixed at low frequencies. This should help prevent EC2 from becoming common and causing the complete breakdown of cooperation. There is some evidence for frequency-dependent cheating in facultative cheaters in *D. discoideum* such that cheating is weaker at higher frequencies (Buttery et al. 2009; Madgwick et al. 2018). Frequency-dependence may be a general limitation on cheating in *D. discoideum*, but the reasons why cheaters do worse at higher frequencies are unclear. It could be that the benefit of cheating decreases as cheaters increase in frequency because there are fewer cooperators to exploit. This could be related to having shorter stalks at higher cheater frequencies.

Cheating can occur whenever the benefits of cooperation can be exploited by those that do not pay the cost of cooperating. However, it is important to understand natural population structure when evaluating the likelihood that a cheating mutation will spread in nature. In this example, obligate social cheaters like these would be unlikely to spread to fixation since relatedness in nature is typically high (Gilbert et al. 2007). This means that obligate cheaters should they arise

in nature will aggregate and develop with clonemates and therefore be unable to fully differentiate into fruiting bodies. In addition, no nonfruiting mutants of *D. discoideum* have ever been found in nature, even though they can be evolved in the lab under conditions of low relatedness (Gilbert et al. 2007; Kuzdzal-Fick et al. 2011a; Inglis et al. 2017; Votaw and Ostrowski 2017). The disadvantages when clonal of not forming spores at all and when not clonal of forming short fruiting bodies are simply too great.

High relatedness can prevent cheaters and cooperators from interacting, whether it is through active processes like kin discrimination or passive processes like population structure. It is thus important to know about natural population structure when determining whether a cheater mutant will spread in nature. For example, different strains of the bacterium *Myxoccocus xanthus* can exhibit strong antagonism against one another (Fiegna and Velicer 2005). However, natural populations of *M. xanthus* are highly structured so mixing between genotypes is unlikely (Vos and Velicer 2008). Furthermore, kin discrimination that segregates genotypes evolves rapidly in this species under laboratory conditions (Rendueles et al. 2015). The costs of cheating to groups that they are a part of is called cheating load, which can be substantial (Travisano and Velicer 2004). Similarly to obligate social cheaters in *D. discoideum*, obligate social cheaters in *M. xanthus* can lead to population collapse when at high frequency (Fiegna and Velicer 2003).

Our results provide evidence for two mechanisms other than high relatedness that could prevent the spread of obligate social cheaters. The first is reduced potential for dispersal and the second is when cheating is frequency-dependent. In this case our obligate social cheater EC2 overrepresents itself in the spores only at low frequencies but not when at high frequencies. This is interesting because EC2 has evolved from a wild ancestor while *fbxA*$^{-}$ is a mutant created in a lab-adapted background strain. For this reason cheating in EC2 may reflect cheating in nature

more closely than cheating in *fbxA*$^{-}$. Frequency-dependent cheating is one way that cooperation can be maintained despite the presence of obligate cheaters and has been found in other microbes such as *P. aeruginosa* (Ross-Gillespie et al. 2007), *M. xanthus* (Velicer et al. 2000), and *Bacillus subtilis* (Pollak et al. 2016). The limits on obligate social cheating other than high relatedness in *D. discoideum* paint a more complete picture of why these cheaters do not spread in nature and cause the collapse of cooperation.

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2.7 Figures

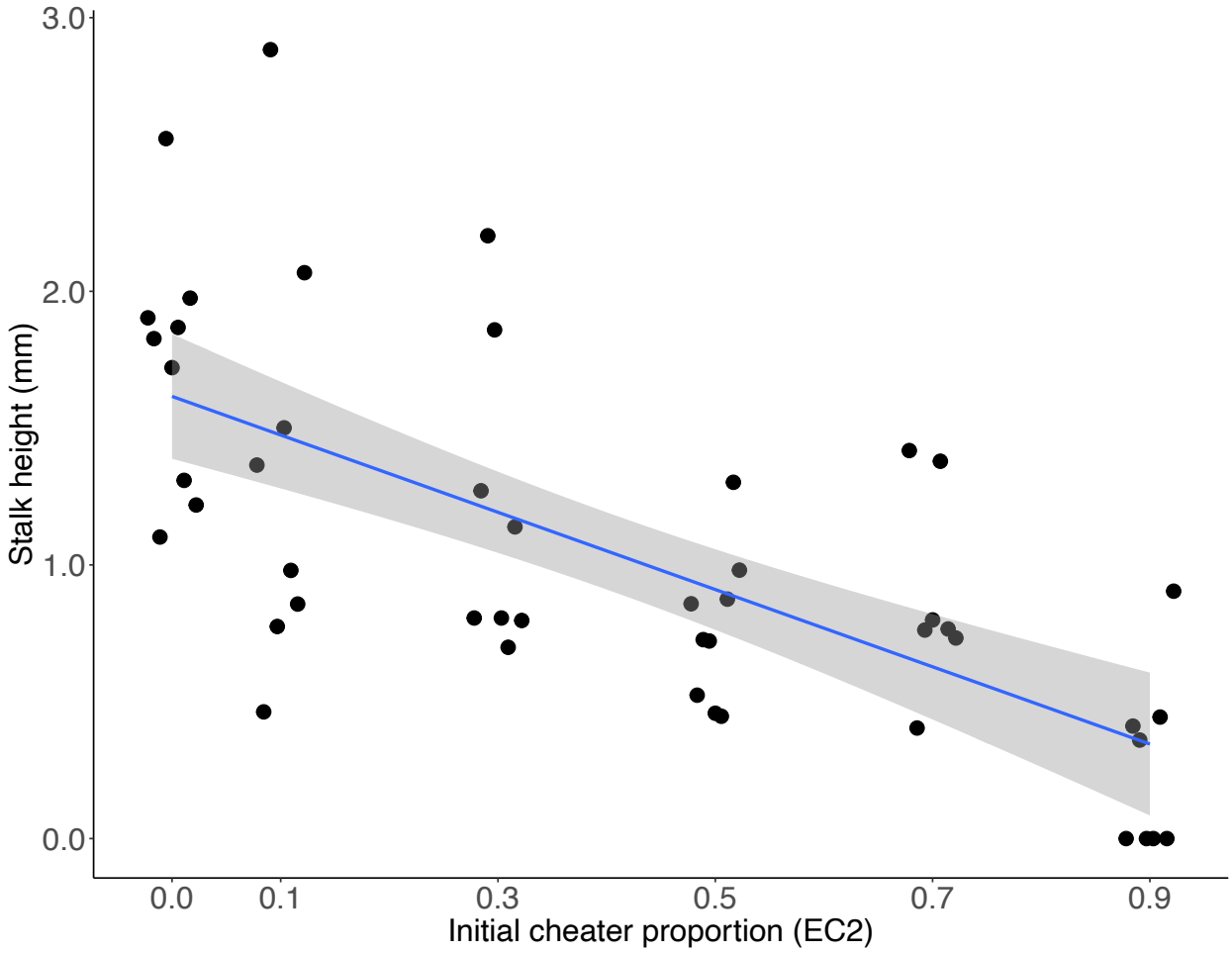

Figure 2.1: Initial cheater proportion predicts stalk height (linear model, DF = 48, t = -6.17, p = 1.37e-07). Regression line is y = -1.34x + 1.6. R-squared = 0.47. Shaded area is 95% CI.

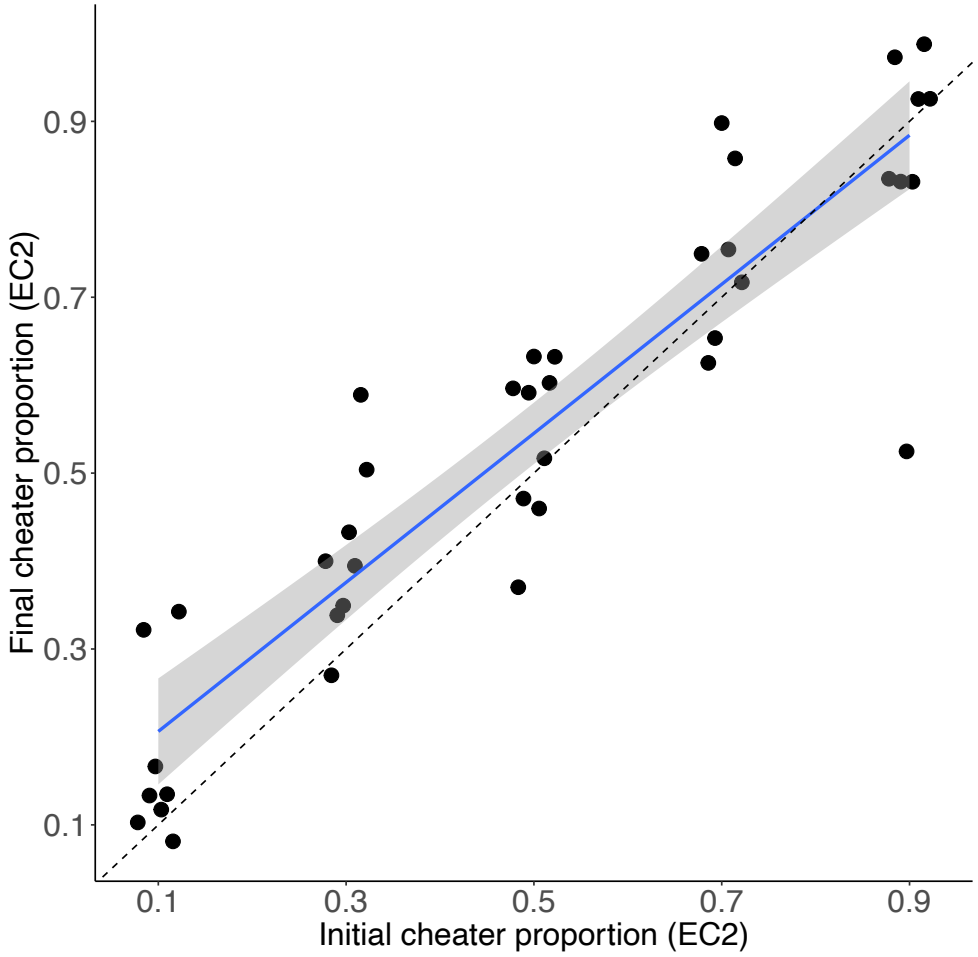

Figure 2.2: Initial cheater proportion predicts final cheater proportion (linear model, DF = 38, t = 13.80, p = 2.23e-16). Regression line is y = 0.85x + 0.12. R-squared = 0.83. Shaded area is 95% CI of the slope of the line. The dashed line shows the null hypothesis of a 1:1 relationship between initial cheater proportion and final cheater proportion.

Chapter 3: The Social Amoeba *Dictyostelium discoideum* rescues *Paraburkholderia hayleyella* but not *P. agricolaris* from interspecific competition

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3.1 Abstract

Bacterial intracellular endosymbionts (hereafter called endosymbionts) can provide benefits for their eukaryotic hosts, but it is often less clear if endosymbionts themselves benefit from these relationships. The social amoeba *Dictyostelium discoideum* is known to associate with three species of *Paraburkholderia* endosymbionts including *P. agricolaris* and *P. hayleyella*. These *Paraburkholderia* are costly to host because they reduce the number of hardy spores produced by *D. discoideum*. However, they can be beneficial because they also allow *D. discoideum* to carry prey bacteria through the dispersal stage to seed new environments where a good bacterial food source may not be available. In laboratory experiments when no other species are present, *P. hayleyella* benefits from associating with *D. discoideum* while *P. agricolaris* does not. However, the presence of other species may influence symbioses like these. We tested if *P. agricolaris* and *P. hayleyella* benefit from the presence of their host in the context of resource competition with *Klebsiella pneumoniae*, *D. discoideum*'s typical laboratory prey. In the absence of *D. discoideum*, *K. pneumoniae* depressed the growth of both *Paraburkholderia* symbionts, consistent with competition between the bacteria. In addition, we found that *P. hayleyella* was harmed more by the presence of *K. pneumoniae* than was *P. agricolaris*. We also found that *P. hayleyella* was rescued from competition with *K. pneumoniae* by the presence of *D. discoideum*

while *P. agricolaris* was not. This may be because *P. hayleyella* is more specialized as an endosymbiont of *D. discoideum;* it has a highly reduced genome compared to *P. agricolaris* and may have lost genes relevant for resource competition outside of its host.

3.2 Introduction

Many eukaryotes associate with bacteria in symbiosis, where two unlike organisms live closely together (DeBary 1879). Our understanding of how these relationships are maintained is incomplete because the costs and benefits of association for symbionts, particularly for intracellular endosymbionts, are understudied compared to the costs and benefits for hosts (Douglas and Smith 1989; Garcia and Gerardo 2014; Mushegian and Ebert 2016). Symbioses are expected to persist for longer when both parties benefit from the interaction (mutualism) than when the symbiont benefits at a cost to the host (parasitism) or when the host benefits at a cost to the symbiont (exploitation). This is because of selection for the host to escape parasitism or the symbiont to escape exploitation (Garcia and Gerardo 2014).

There are many examples of parasitism, but for cases where the host benefits it is often less clear whether they are more consistent with mutualism or exploitation. The mutualistic explanation is supported in some systems in which the endosymbionts benefit (Kuykendall 1989; Lee and Ruby 1994; Storelli et al. 2018; Iwai et al. 2019). But in others, endosymbiont population sizes are controlled by the host and it is unclear whether this cost is sufficiently compensated by other benefits (Johnson et al. 2007; Lowe et al. 2016; Whittle et al. 2021).

Symbioses are not strictly categorical and can range along the spectrum from antagonistic to mutualistic depending on the costs and benefits of symbiosis for each partner (Lewis 1985) as well as the degree to which the fitness of one partner depends on the fitness of the other (Queller 2011). For example, obligate endosymbionts, which cannot live outside their hosts, are expected

to have their fitness interests more aligned with their hosts than facultative endosymbionts that can live free of hosts (Douglas and Smith 1989; Frank 1997; Garcia and Gerardo 2014; Mushegian and Ebert 2016). However, this may be true only early in the evolution of a given symbiosis because the loss of independent reproduction can result in the eventual extinction of an endosymbiont (Keeling and McCutcheon 2017; Husnik and Keeling 2019).

Furthermore, the costs and benefits of symbiosis can change depending on ecological factors such as environmental context (Bronstein 1994; Thompson 1994; Chamberlain et al. 2014) and the presence of other species (Wootton 1994; Rudgers and Strauss 2004). To understand how symbioses are maintained, it is thus important to test the costs and benefits of symbiosis across ecological contexts. Here we investigate the effect of a model eukaryote, *Dictyostelium discoideum*, on the fitness of two of its facultative endosymbiotic bacteria species, *Paraburkholderia agricolaris* and *P. hayleyella*, when a competitor bacterium, *Klebsiella pneumoniae,* is present or absent.

D. discoideum is a social amoeba that preys on bacteria in forest soils (Swanson et al. 1999; Kessin 2001). Starvation initiates the social cycle, wherein individual amoebae aggregate into a multicellular slug that travels to a new location and further develops into a multicellular fruiting body. During this process, about 20% of the cells die to become a stalk that holds up a ball of reproductive spores called the sorus for dispersal (Kessin 2001; smith et al. 2014; Medina et al. 2019). After dispersal, spores hatch in new environments as vegetative amoebae and resume preying upon bacteria.

While *D. discoideum* transiently associates with many bacterial species, three species of the genus *Paraburkholderia* - *P. agricolaris*, *P. hayleyella* and *P. bonniea* - can stably associate with

the amoeba through multiple rounds of the social cycle (Brock et al. 2018, 2020; Haselkorn et al. 2019). These species can be found in spores and cells of *D. discoideum* and can also be cultured outside the host (Shu et al. 2018a; Khojandi et al. 2019). About a quarter of wild-collected *D. discoideum* clones are infected with these *Paraburkholderia*. Carrying *Paraburkholderia* is costly to *D. discoideum* in food-rich contexts because the bacteria reduce the number of spores *D. discoideum* produced as well as the distance that *D. discoideum* slugs traveled (Brock et al. 2016; Haselkorn et al. 2019). However, these bacteria can benefit the amoebae when food is scarce. While they are not themselves edible, *Paraburkholderia* can induce secondary carriage of other species of bacteria which are better food sources for *D. discoideum* such as *Klebsiella pneumoniae* (Haselkorn et al. 2019; Khojandi et al. 2019). When *D. discoideum* spores disperse to an environment without a food source, amoebae can prey on populations of bacteria derived from those they carried with them (Brock et al. 2011; DiSalvo et al. 2015). Simulations lend support to the idea that infection can provide long-term benefits to the amoebae when bacterial prey are patchily distributed in the environment (Scott et al. 2022).

These three species of *Paraburkholderia* represent two independent transitions to endosymbiosis with *D. discoideum*, once in *P. agricolaris* and once in *P. hayleyella* plus *P. bonniea*, which are each other's closest relatives (Haselkorn et al. 2019). We focus on the differences between *P. agricolaris* and *P. hayleyella* because they are the most common in nature and because *P. hayleyella* and *P. bonniea* are similar to each other (Brock et al. 2020; Noh et al. 2022). *P. agricolaris* and *P. hayleyella* also make an interesting contrast because *P. hayleyella* is more like an obligate symbiont than *P. agricolaris*. For example, *P. hayleyella* has a sharply reduced genome, half the size of that of *P. agricolaris*, as well as a lower GC content (Brock et al. 2020; Noh et al. 2022). Both these characteristics are common in long-term endosymbionts in general,

both mutualists and pathogens, as well as endosymbionts of protists in particular (McCutcheon and Moran 2012; George et al. 2020).

In addition, *P. hayleyella* may depend more on *D. discoideum* for its fitness than *P. agricolaris* does. When cultured in soil, *P. hayleyella* has higher fitness in association with *D. discoideum* than alone whereas *P. agricolaris* does not (Garcia et al. 2019). Both *P. agricolaris* and *P. hayleyella* swim towards *D. discoideum* supernatant, and *P. hayleyella* prefers amoebae that are currently or had been previously infected while *P. agricolaris* does not share this preference (Shu et al. 2018b).

Symbioses like the one between *D. discoideum* and *Paraburkholderia* do not exist in isolation. *D. discoideum* cannot subsist on *P. agricolaris* or *P. hayleyella* alone. Food bacteria must also be present and may compete with these *Paraburkholderia* species for resources. In the presence of *D. discoideum*, a higher titer of the food bacterium *Klebsiella pneumoniae* reduces the fitness of both *P. agricolaris* and *P. hayleyella* (Scott et al. 2022). *P. hayleyella* in particular may be a poor resource competitor because it cannot utilize as many carbon sources as *P. agricolaris* (Brock et al. 2020). We chose to use *K. pneumoniae* as a model third-party, both as a competitor for *Paraburkholderia* and food for *D. discoideum* because *K. pneumoniae* and *Paraburkholderia* can co-colonize *D. discoideum* cells (Brock et al. 2011; Shu et al. 2018a) and be co-dispersed through the spores (Brock et al. 2011). In addition, *K. pnemoniae* is a well-studied bacterial food for culturing *D. discoideum* (Kessin 2001).

For our experiments we first used 5% of each *Paraburkholderia* species relative to 95% *K. pneumoniae* because this ratio has previously been shown to support *D. discoideum* growth. We repeated the experiments with 50% *P. agricolaris* relative to *K. pneumoniae* in order to

accentuate the effects of competition between the species, our logic being that at more equal higher frequencies there are more opportunities for direct interactions between the bacterial species. We did not do the 50% experiments with *P. hayleyella* because *P. hayleyella* becomes too toxic to *D. discoideum* at that level.

We hypothesized first that both *P. agricolaris* and *P. hayleyella* reach lower abundances in the presence of *K. pneumoniae* relative to when they are cultured alone due to competition between the species. Second, we hypothesized that competition with *K. pneumoniae* would harm *P. hayleyella* more than *P. agricolaris*, reflected in a greater drop in abundance, because *P. hayleyella* is a poorer competitor due to its reduced genome size and metabolic capability compared to *P. agricolaris*. Third, because *D. discoideum* consumes *K. pneumoniae*, we hypothesized that *P. agricolaris* and *P. hayleyella* would have higher abundance when additionally cultured with *D. discoideum*. Fourth, we hypothesized that *P. hayleyella* would receive a greater benefit from the presence of *D. discoideum* than *P. agricolaris* because it is a poorer competitor and has more to gain from having its competitors consumed.

3.3 Methods

3.3.1 Culturing and maintenance of *D. discoideum*, *Paraburkholderia*, and *K. pneumoniae*

To grow stocks of non-pathogenic *K. pneumoniae* KpGe (Dicty Stock Center, dictybase.org), we streaked them out from 20% glycerol freezer stocks. We made SM/5 agar plates [2 g glucose (Fisher Scientific), 2 g Bacto Peptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g MgSO4 * 7H2O (Fisher Scientific), 1.9 g KH2PO4 (Sigma-Aldrich), 1 g K2HPO4 (Fisher Scientific), and 15 g agar (Fisher Scientific) per liter] and incubated the bacteria for 2 days at room temperature. We collected bacteria, diluted in KK2 buffer [2.25 g KH2PO4 (Sigma-Aldrich) and 0.67 g K 2HPO4

(Fisher Scientific) per liter] to 1.5 OD_{600} as measured by an Eppendorf BioPhotometer and kept stocks at 4°C until use. For the competition experiment we grew fresh *K. pneumoniae* from freezer stocks and repeated the above process as needed, keeping stocks at 4°C until use (1 day).

We grew *D. discoideum* clones from 20% glycerol freezer stocks on SM/5 agar plates with 200 µl of 1.5 OD_{600} *K. pneumoniae* as a food source and incubated plates for 7 days at room temperature under constant light to allow amoebas to complete the social cycle and form fruiting bodies. We collected spores from fruiting bodies and then cured them of *Paraburkholderia* infection as previously described (DiSalvo et al. 2015). These cured spores were frozen and revived when needed using the same procedure for use in the competition experiments.

For the competition experiment we grew *Paraburkolderia* isolates from 20% glycerol freezer stocks by streaking out bacteria on SM/5 agar plates and incubating for 3-4 days at room temperature. We collected bacteria, diluted in KK2 buffer to 1.5 OD_{600} and kept stocks at 4°C for no more than one day before using.

3.3.2 Competition experiment

For the competition experiment we used three treatments: *Paraburkholderia* alone, *Paraburkholderia* with *K. pneumoniae*, and *Paraburkholderia* with *K. pneumoniae* and *D. discoideum*. We used five clones of *P. agricolaris* and five clones of *P. hayleyella*, one clone per treatment at a time. We used a single competitor clone of *K. pneumoniae*, KpGe, for these experiments. For the treatment including *D. discoideum* we matched the *Paraburkholderia* clone to the *D. discoideum* clone from which it was originally isolated to keep as much of the natural context as possible.

For each clone-by-treatment combination we made a premix mix consisting of 5% 1.5 OD_{600} *P. agricolaris* or *P. hayleyella*, 95% 1.5 OD_{600} *K. pneumoniae*, and about 1600 *D. discoideum* spores. Treatments that lacked *D. discoideum* or *K. pneumoniae* had the volume made up with KK2 buffer. We distributed this premix among the eight inner wells of a 24-well plate containing 1 ml of SM/5 agar per well for eight technical replicates. We used only the inner 8 wells of each plate because we found that the outer wells are susceptible to drying out over the length of time it takes *D. discoideum* to make fruiting bodies. For *P. agricolaris* we repeated this experiment with 50% *P. agricolaris* and 50% *K. pnemoniae* in addition to the experiment with 5% *P. agricolaris* and 95% *K. pneumoniae* as described above.

We incubated plates at room temperature for 7 days at room temperature under constant overhead light to allow *D. discoideum* to complete its social cycle. We then collected the contents of each well into 750 µl of KK2 for quantification by qPCR. We treated each sample with 50 µM PMAxx (Biotium) to prevent cell-free DNA from inflating counts of *Paraburkholderia* cells as previously described (Garcia et al. 2019). We extracted DNA from each sample using the Zymo Research Quick-DNA Fecal/Soil Microbe Kit according to the manufacturer's protocol, processing each sample in a Disruptor Genie at 3,000 rpm for 20 minutes. We quantified the abundance of *P. agricolaris* and *P. hayleyella* using qPCR as previously described (Garcia et al. 2019). We excluded one *P. hayleyella* clone, Ph69, from the analyses because it did not grow or grew very little under the experimental conditions when cultured alone and with *K. pneumoniae*.

3.3.3 Statistical analyses

We analyzed the data from the 5% *Paraburkholderia* experiment with generalized linear models (GLM) using the glm command in R v 4.2.2. Specifically, we asked two questions: 1) whether

the presence of a second bacterium (*K. pneumoniae)* suppressed the population size of *Paraburkholderia* due to competition, and 2) whether the presence of *D. discoideum* with *Paraburkholderia* and *K. pneumoniae* rescued *Paraburkholderia* from the effects of competition. For the first question, we modeled the effect of the *Paraburkholderia* alone vs. *Paraburkholderia* with *K. pneumoniae* treatments and *Paraburkholderia* species on *Paraburkholderia* population size. For the second question, we modeled the effect of *Paraburkholderia* with *K. pneumoniae* vs. *Paraburkholderia* with *K. pneumoniae* and *D. discoideum* treatments and *Paraburkholderia* species on *Paraburkholderia* population size. Both GLMs used a quasipoisson error distribution to correct for overdispersion. Stepwise model selection and significance tests for each term were performed using likelihood ratio tests with F tests for both models. The emmeans function from the emmeans package was used to calculate estimated marginal means (reported below with standard error) and pairwise contrasts with the Tukey method of adjustment for multiple comparisons (Lenth 2022).

We also analyzed the data from the 50% *P. agricolaris* experiment with two GLMs. We followed the framework above and used separate GLMs to address each question. However, here we modeled the effect of *P. agricolaris* strain (instead of *Paraburkholderia* species) and treatment on *P. agricolaris* population size using a quasipoisson error distribution to correct for overdispersion. Stepwise model selection, significance tests, and contrasts were done as above.

3.4 Results

For our first question, whether *K. pneumoniae* suppresses the population size of *Paraburkholderia*, we found that all strains of both *Paraburkholderia* species had a decrease in population size when cocultured with *K. pneumoniae* (Fig. 3.1). Both treatment (likelihood ratio test, $F = 189.21$, $df = 1$, $p < 2.2 \times 10^{-16}$) and *Paraburkholderia* species (likelihood ratio test, $F =$

117.95, $df = 1$, $p < 2.2 \times 10^{-16}$) had a significant effect on *Paraburkholderia* population size. Furthermore, there was a significant interaction between treatment (with or without *K. pneumoniae*) and *Paraburkholderia* species (likelihood ratio test, $F = 5.6722$, $df = 1$, $p = 0.0186$). *P. hayleyella* had both a significantly smaller monoculture population size than *P. agricolaris* ($9.2 \times 10^3 \pm 1.4 \times 10^3$ vs. $3.9 \times 10^4 \pm 2.7 \times 10^3$ cells/μL; Tukey adjusted contrast, $p < 0.0001$) and was significantly more harmed by *K. pneumoniae* than *P. agricolaris* was ($2.2 \times 10^2 \pm 2.2 \times 10^2$ vs. $5.9 \times 10^3 \pm 1.0 \times 10^3$ cells/μL; Tukey-adjusted contrasts, $p = 0.0012$; Fig 3.3, Table 3.1).

For our second question, whether *D. discoideum* rescues *Paraburkholderia* from competition with *K. pneumoniae*, we found variation in the effect of *D. discoideum* both within and between *Paraburkholderia* species (Fig. 3.2). All but one *P. hayleyella* strain had an increase in population when grown with *K. pneumoniae* and *D. discoideum*, but only two of five *P. agricolaris* strains showed a similar increase (Fig. 3.2). *Paraburkholderia* species (likelihood ratio test, $F = 134.54$, $df = 1$, $p < 2.2 \times 10^{-16}$) and the interaction between *Paraburkholderia* species and treatment (likelihood ratio test, $F = 6.5866$, $df = 1$, $p = 0.0113$) had significant effects on *Paraburkholderia* population size, but treatment did not (likelihood ratio test, $F = 0.0124$, $df = 1$, $p = 0.9116$). We found that *P. hayleyella* gets this rescue effect ($2.2 \times 10^2 \pm 1.3 \times 10^2$ vs. $9.1 \times 10^2 \pm 2.6 \times 10^2$ cells/μL; Tukey-adjusted contrasts, $p = 0.0262$) while *P. agricolaris* does not ($5.9 \times 10^3 \pm 5.8 \times 10^2$ vs. $5.4 \times 10^3 \pm 5.7 \times 10^2$ cells/μL; Tukey-adjusted contrasts, $p = 0.5699$, Table 3.1). Although we saw a general pattern of *Paraburkholderia* suppression due to competition with *K. pneumoniae*, there was variation in the strength of competition as well as the effect of *D. discoideum* on the competitive interaction at the strain-level of both *Paraburkholderia* species (Figs 3.4 and 3.5).

For our main experiment we used 5% *Paraburkholderia* to 95% *K. pneumoniae*. However, we also repeated this with 50% *P. agricolaris* to 50% *K. pneumoniae* to test whether accentuating the effects of competition would increase the benefit *P. agricolaris* receives from associating with *D. discoideum*. Our reasoning was that at a higher relative frequency there would be more opportunities for direct interactions between *P. agricolaris* and *K. pneumoniae*. We did not repeat this with *P. hayleyella* because we have found most *P. hayleyella* strains are toxic to *D. discoideum* at high concentrations (Khojandi 2019). Overall, we found similar results to our 5% *P. agricolaris* experiment, specifically that *P. agricolaris* had significantly reduced abundance when cultured with *K. pneumoniae* (likelihood ratio test for treatment, $F = 81.928$, $df = 1$, $p < 1.68 \times 10^{-13}$; Table 3.2) but did not recover when cultured with *D. discoideum* (likelihood ratio test for treatment, $F = 3.789$, $df = 1$, $p = 0.0561$; Fig. 3.6, Table 3.2). However, there were strain-level differences between the 5% and 50% treatments: some clones even had the reverse pattern compared to the 5% treatment. For example, PaNC21 was more harmed by the addition of *D. discoideum* in the 5% treatment but instead benefited significantly in the 50% treatment (Tukey-adjusted contrast, $p = 0.0229$; Fig 3.7). Pa317s did very poorly when *D. discoideum* was added in the 5% treatment, but not nearly as badly in the 50% treatment (Fig 3.7). The result was not always beneficial to *P. agricolaris* in the 50% treatment though. Pa31 benefited in the 5% treatment when *D. discoideum* was added but not in the 50% treatment (Fig 3.7).

3.5 Discussion

The costs and benefits of endosymbiosis for eukaryotic hosts are well characterized, but less is known about these factors for bacterial endosymbionts. Knowing the average costs and benefits of association for endosymbionts across ecological contexts is important for understanding how these relationships change over evolutionary time. We investigated the costs and benefits for two

species of endosymbiotic *Paraburkholderia*, *P. agricolaris* and *P. hayleyella*, in the context of interspecific competition. We cultured each *Paraburkholderia* species alone, with a competitor bacterium *K. pneumoniae*, and with both *K. pneumoniae* and *D. discoideum*, a natural host of *P. agricolaris* and *P. hayleyella* that also preys on *K. pneumoniae*. For each treatment, we used qPCR to measure the abundance of *P. agricolaris* or *P. hayleyella*. *P. hayleyella* seems to be further along the path to becoming an obligate endosymbiont, one that cannot live without its host, than *P. agricolaris*. *P. hayleyella* has a reduced genome and GC content compared to *P. agricolaris* (Brock et al. 2020; Noh et al. 2022), grows more poorly than *P. agricolaris* outside of its host (Garcia et al. 2019), benefits more from the presence of its host (Garcia et al. 2019), and swims towards its host while *P. agricolaris* does not (Shu et al. 2018).

Both *P. agricolaris* and *P. hayleyella* had the highest abundance when they were cultured independently, compared to when they were co-cultured with *K. pneumoniae* or with both *K. pneumoniae* and *D. discoideum* (Fig. 3.3). Consistent with the idea that *P. agricolaris* is better at utilizing non-host resources than *P. hayleyella*, we found that *P. agricolaris* reaches significantly larger population sizes than *P. hayleyella* does on its own (Fig. 3.3). When each species was cocultured with a competitor, *K. pneumoniae*, both species reached significantly lower final abundances than they did when cultured alone (Fig. 3.3). This supports our hypothesis that each *Paraburkholderia* species competed with *K. pneumoniae*. We also found support for our hypothesis that *P. hayleyella* was harmed significantly more by the presence of *K. pneumoniae* than *P. agricolaris* (Figs. 3.1 and 3.3). This may be because *P. hayleyella* has reduced metabolic capacity compared to *P. agricolaris* which could make it a poorer competitor for resources outside of the host (Brock et al. 2020; Noh et al. 2022).

Interestingly we also found that *D. discoideum* significantly increased *P. hayleyella* abundance but not *P. agricolaris* in the presence of *K. pneumoniae* (Figs. 3.2 and 3.3). *P. hayleyella* has features similar to long-term endosymbionts in other systems such as a reduced genome size and lowered GC content whereas *P. agricolaris* does not, so it may rely more on its host for fitness than *P. agricolaris* does (Brock et al. 2020; Noh et al. 2022). Our results support this idea, as do results from another study that showed that *P. hayleyella* also attained higher abundance with *D. discoideum* in the absence of *K. pneumoniae* whereas *P. agricolaris* did not (Garcia et al. 2019).

While study of a another predatory protist and endosymbiont found that *Paramecium bursaria* consumed competitors of its algal endosymbiont (Iwai et al. 2019), it is not clear if the same process explains our results. Since *D. discoideum* does prey on *K. pneumoniae*, it could be that *P. hayleyella* benefits outside of the host because *D. discoideum* consumes its competitors. However, *P. agricolaris* should get a similar benefit for the same reason, which on average it does not. That said, *P. agricolaris* is already a stronger competitor than *P. hayleyella* in that it is not harmed as much by the presence of *K. pnemoniae* in the first place. So it may be that only a weaker competitor benefits from having its competitors consumed.

Alternatively, *P. hayleyella* may gain nutritional benefits from colonizing *D. discoideum* that *P. agricolaris* does not receive. Since *P. hayleyella* can attain higher population sizes in association with *D. discoideum* than on its own while *P. agricolaris* does not, this may be because *P. hayleyella* can use *D. discoideum* as a food source better than *P. agricolaris* can (Garcia et al. 2019). This would be consistent with greater adaptation to *D. discoideum* in *P. hayleyella* than *P. agricolaris*.

Another important aspect to note is variation across strains in the costs of having a competitor *K. pneumoniae* present and in the benefit from associating with *D. discoideum*, particularly in *P. agricolaris* (Figs. 3.4 and 3.5). In order to preserve the natural context of the interactions we paired each clone of *D. discoideum* with the *Paraburkholderia* strain that it was isolated with from nature, five pairs for *P. agricolaris* and four pairs for *P. hayleyella*. It is interesting, but not unexpected, that there is variation within each species in the costs and benefits they receive from endosymbiosis. There is also variation between strains within each *Paraburkholderia* species in how they affect their *D. discoideum* hosts (Miller et al. 2020). In addition, there is more variation among strains of *P. agricolaris* than *P. hayleyella*. This may be because *P. hayleyella* has adapted more to endosymbiosis with *D. discoideum* than *P. agricolaris* has.

The differences between the 5% and 50% *P. agricolaris* experiments may be due to the different frequency of competitive interactions with *K. pneumoniae*, but there are other explanations as well. *P. agricolaris* is somewhat toxic to *D. discoideum,* and at 50% frequency not all of our replicates were able to fruit, which we excluded from the analysis. It may be that some *P. agricolaris* are able to do better under these conditions because *D. discoideum* is weakened and easier to exploit. Or if *D. discoideum* is weakened or dead it may produce fewer antimicrobial compounds that inhibit the growth of *P. agricolaris* and *P. hayleyella* as it does in liquid culture (Garcia et al. 2019). This may interact with predation by *D. discoideum* on *K. pneumoniae* in a way that varies by clone.

This work highlights the importance of using multiple naturally collected clones, not only multiple species, in the study of the evolution and ecology of intracellular endosymbiosis. Future work on this topic should continue to focus on the fitness of endosymbionts as well as hosts, sampling natural variation across ecological contexts. This will aid in our understanding of the

relationships between bacteria and eukaryotes, including endosymbiosis, the evolution of organelles, and the major transitions in evolution.

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3.7 Figures

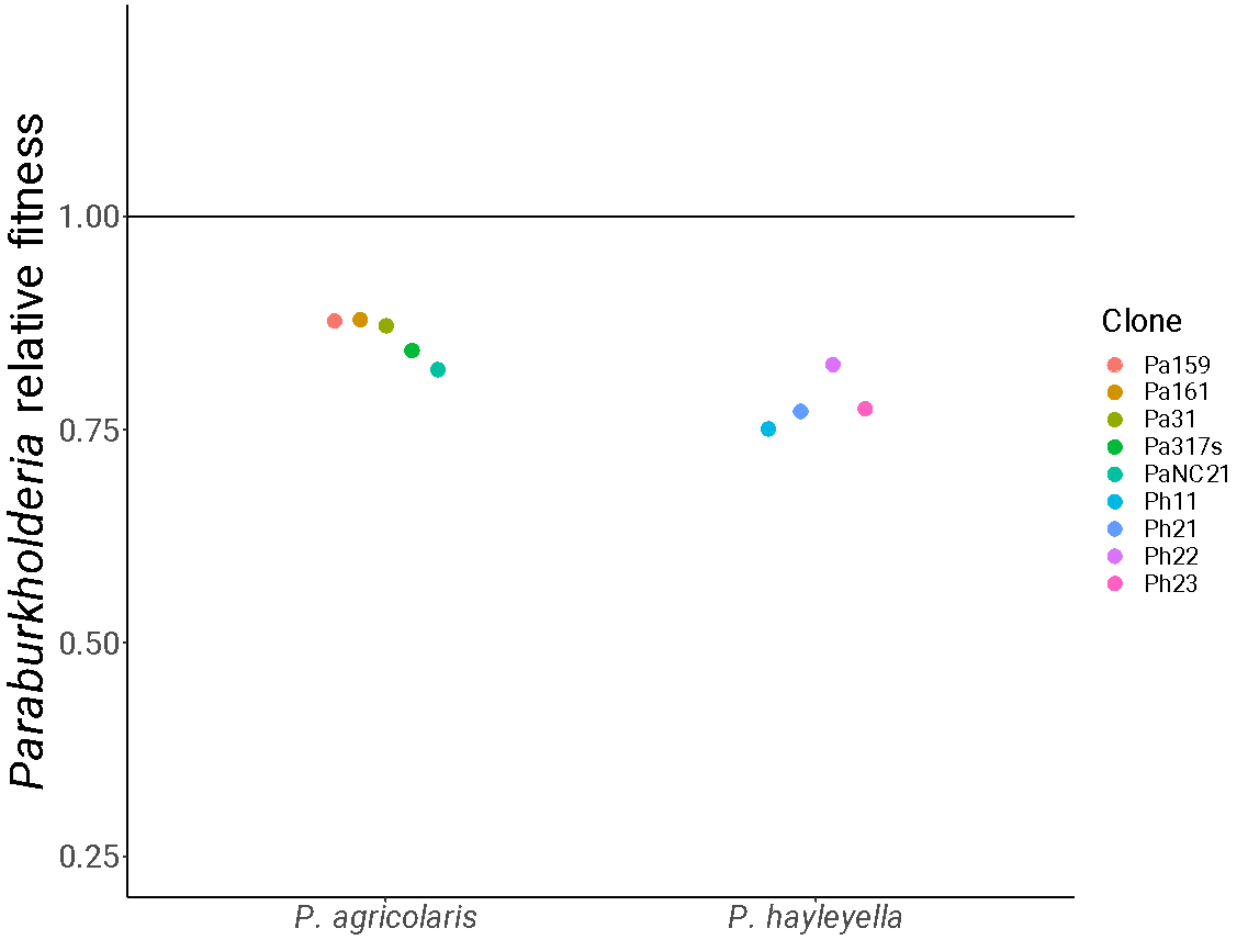

Figure 3.1. Competition with *K. pneumoniae* harms both *P. hayleyella* and *P. agricolaris*. Each point represents results for a single clone, the difference between the mean of 8 technical replicates of *Paraburkholderia* cultured alone and 8 technical replicates of *Paraburkholderia* cultured with *K. pneumoniae* at a 5:95 ratio. *Paraburkholderia* relative fitness is the abundance

of *Paraburkholderia* in the treatment where it was cultured with *K. pneumoniae* divided by the abundance of *Paraburkholderia* in the alone treatment.

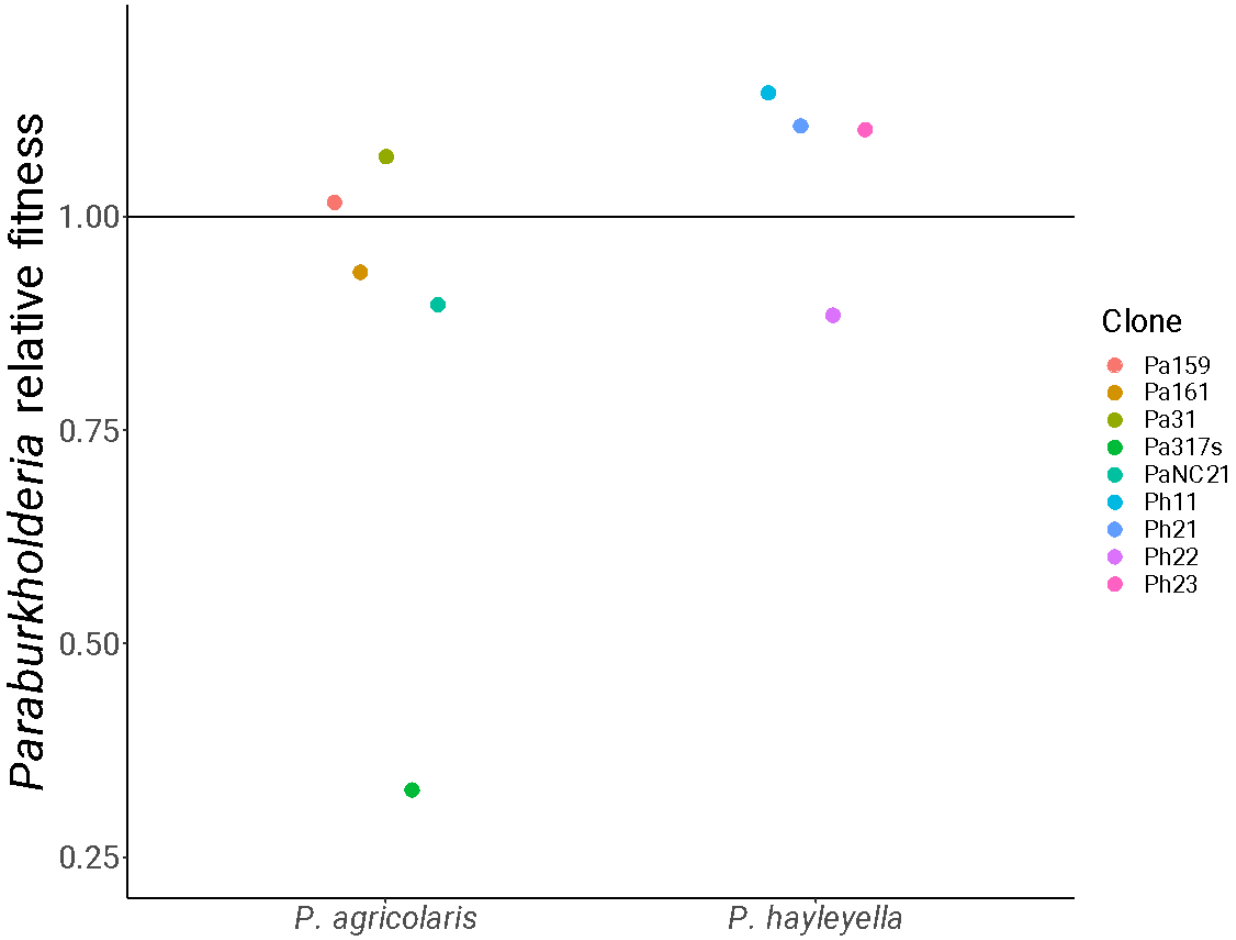

Figure 3.2. *D. discoideum* rescues some clones of *P. hayleyella* and *P. agricolaris* from interspecific competition with *K. pneumoniae*. Each point represents results for a single clone, the difference between the mean of 8 technical replicates of *Paraburkholderia* cultured with *K. pneumoniae* at a 5:95 ratio and 8 technical replicates of *Paraburkholderia* cultured with *K.*

pneumoniae at a 5:95 ratio with *D. discoideum* present. *Paraburkholderia* relative fitness is the abundance of *Paraburkholderia* in the treatment where it was cultured with *K. pneumoniae* and *D. discoideum* divided by the abundance of *Paraburkholderia* in the *Paraburkholderia* and *K. pneumoniae* treatment.

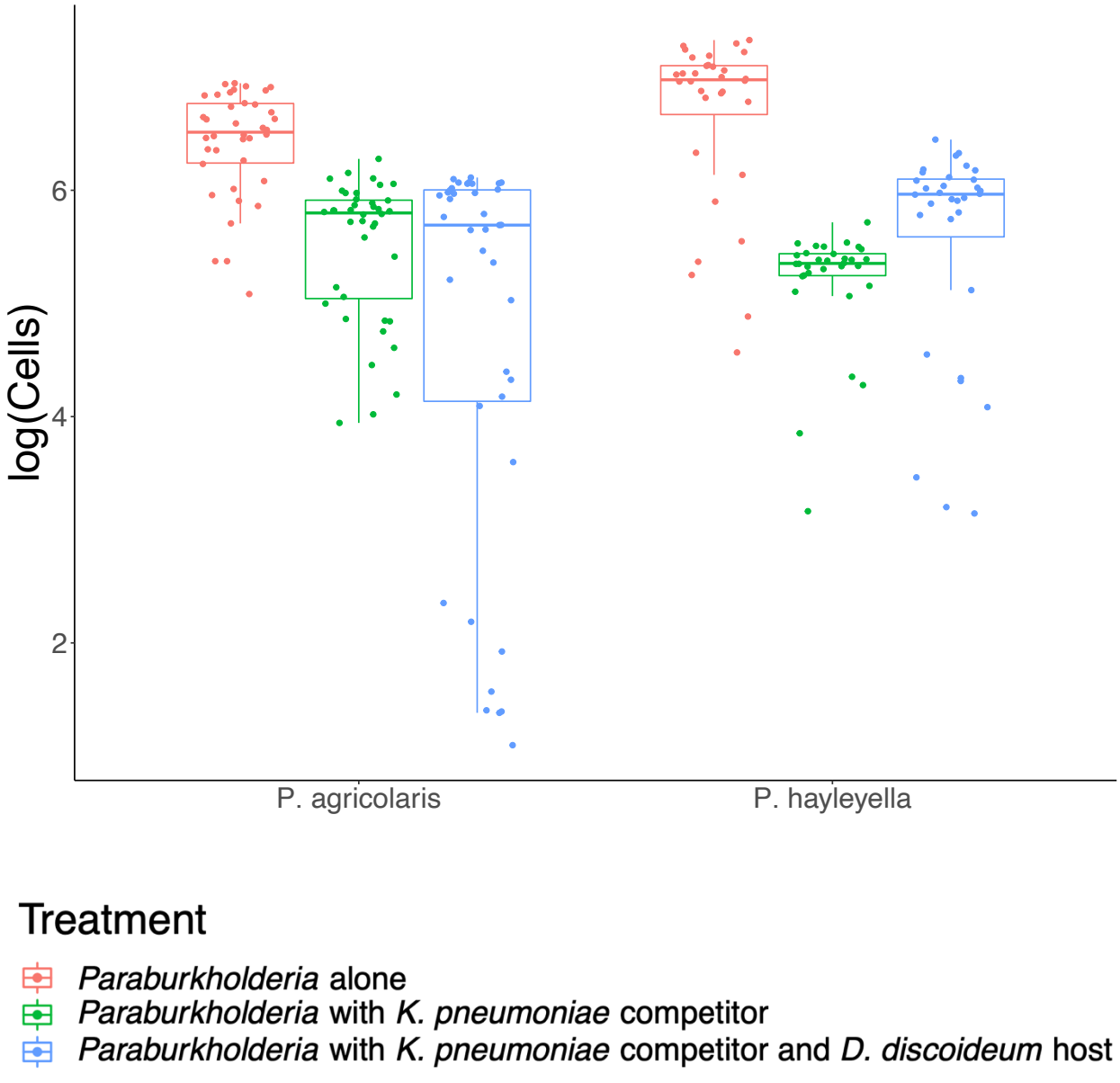

Figure 3.3. *P. hayleyella* is harmed more than *P. agricolaris* by competition with *K. pneumoniae* and recovers more fitness in the presence of *D. discoideum*. Ratio of *Paraburkholderia* to *K. pneumoniae* is 5:95.

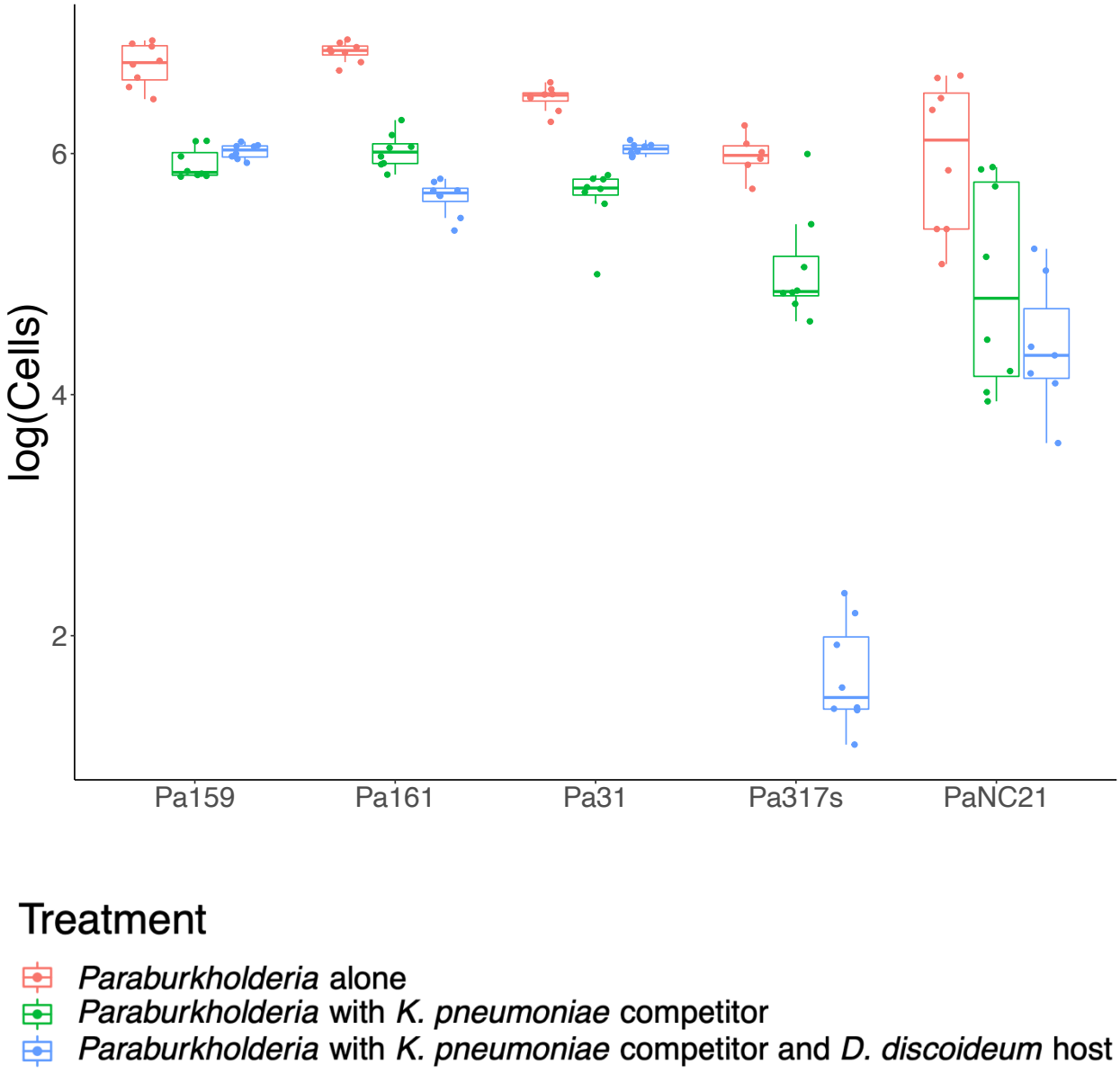

Figure 3.4. Variation in rescue effect of *D. discoideum* across strains of *P. agricolaris*. All strains have lower fitness in the presence of the competitor *K. pneumoniae*. When *D. discoideum* is also present some strains recover part of their fitness while others do worse or no differently. Ratio of *Paraburkholderia* to *K. pneumoniae* is 5:95.

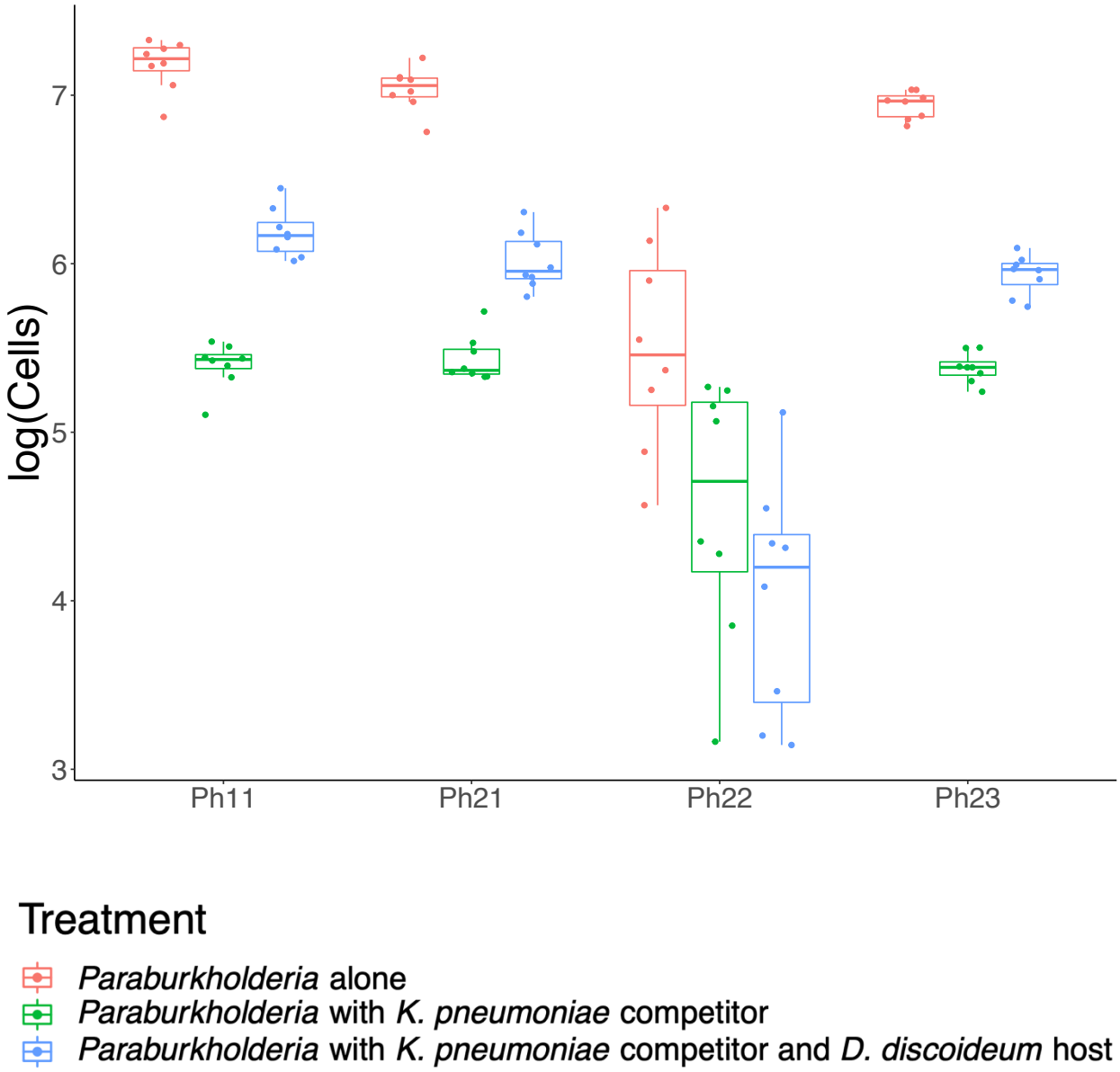

Figure 3.5. Variation in rescue effect of *D. discoideum* across strains of *P. hayleyella*. All strains have lower fitness in the presence of the competitor *K. pneumoniae*. When *D. discoideum* is also present most strains recover part of their fitness. Ratio of *Paraburkholderia* to *K. pneumoniae* is 5:95.

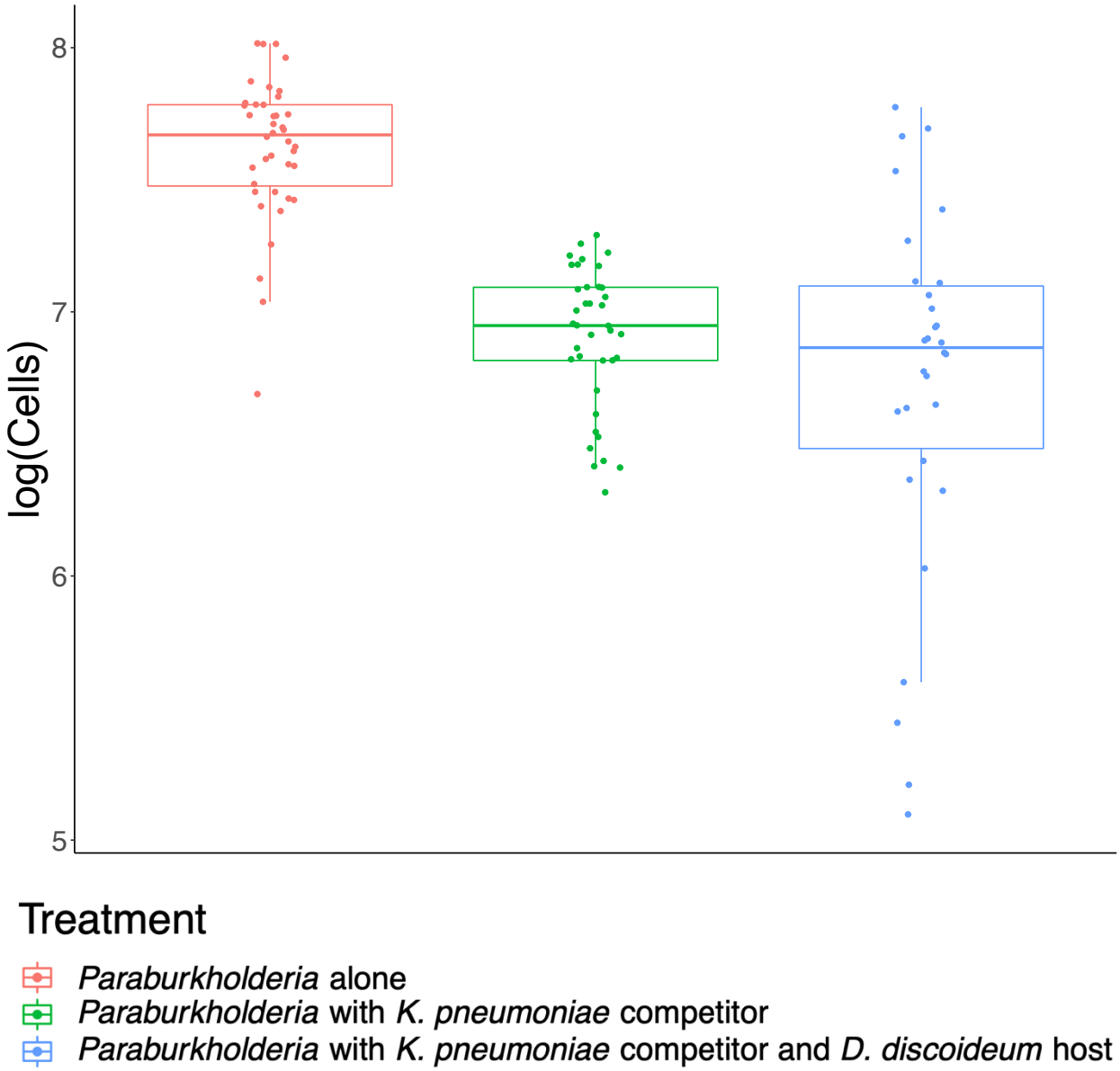

Figure 3.6. *P. agricolaris* is harmed competition with *K. pneumoniae* and does not recover in the presence of *D. discoideum*, similarly to the 5:95 ratio experiment. Ratio of *Paraburkholderia* to *K. pneumoniae* is 50:50.

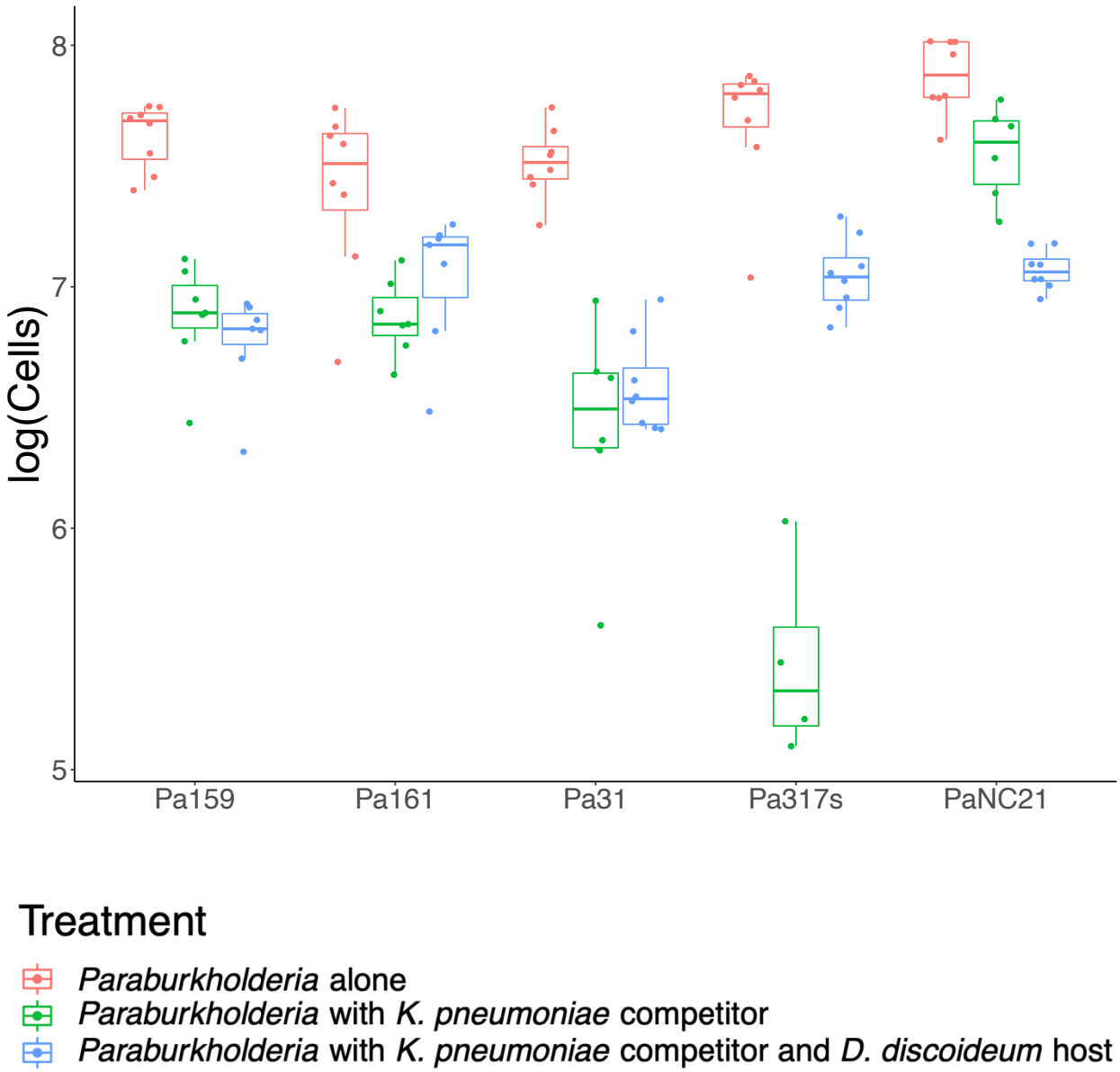

Figure 3.7. Variation in rescue effect of *D. discoideum* across strains of *P. agricolaris*. All strains have lower fitness in the presence of the competitor *K. pneumoniae*. When *D. discoideum* is also present some strains recover part of their fitness while others do worse or no differently. Ratio of *Paraburkholderia* to *K. pneumoniae* is 50:50.

Table 3.1: Parameters from generalized linear models testing the effect of treatment and *Paraburkholderia* species on *Paraburkholderia* population. This data is from the experiment in which *Paraburkholderia* was mixed with *K. pneumoniae* in 5:95 ratio. Both *Paraburkholderia* species, *P. agricolaris* and *P. hayleyella*, were included in this experiment.

A) Test for effect of competition (*Paraburkholderia* alone vs. *Paraburkholderia* with *K. pneumoniae*) Model: *Paraburkholderia* Population ~ Treatment**Paraburkholderia* Species			
Term	*df*	*F*	*p*
Treatment	1	189.21	$< 2.2x10^{-16}$
Paraburkholderia Species	1	117.95	$< 2.2x10^{-16}$
Treatment***Paraburkholderia* Species	1	5.6722	0.0186
Estimated marginal means	Cells/μL	Standard error	
P. agricolaris alone treatment	38,945	2,682	
P. agricolaris + *K. pneumoniae* treatment	5,869	1,015	

P. hayleyella alone treatment	9,154	1,417	
P. hayleyella + *K. pneumoniae* treatment	218	219	
Pairwise contrasts	*Z* ratio	*p*	
P. agricolaris vs. *P. hayleyella* alone treatment	8.548	<0.0001	
P. agricolaris vs. *P. hayleyella* *K. pneumoniae* treatment	3.235	0.0012	
B) Test for competition rescue by *D. discoideum* (*Paraburkholderia* with *K. pneumoniae* vs. *Paraburkholderia* with *K. pneumoniae* & *D. discoideum*) Model: *Paraburkholderia* Population ~ Treatment**Paraburkholderia* Species			
Term	*df*	*F*	*p*
Treatment	1	0.0124	0.9116
Paraburkholderia Species	1	134.54	$< 2.2x10^{-16}$
Treatment**Paraburkholderia* Species	1	6.5866	0.0113
Estimated marginal means	Cells/μL	Standard error	
P. agricolaris + *K. pneumoniae* treatment	5869	567	
P. agricolaris + *K. pneumoniae* + *D. discoideum* treatment	5407	567	
P. hayleyella + *K. pneumoniae* treatment	218	126	

P. hayleyella + *K. pneumoniae* + *D. discoideum* treatment	908	257	
Pairwise contrasts	*Z* ratio	*p*	
P. agricolaris, *K. pneumoniae* vs. *K. pneumoniae* + *D. discoideum* treatment	-0.568	0.5699	
P. hayleyella, *K. pneumoniae* vs. *K. pneumoniae* + *D. discoideum* treatment	2.223	0.0262	

Table 3.2: Parameters from generalized linear models testing the effect of treatment and *P. agricolaris* strain on *P. agricolaris* population. This data is from the experiment in which *Paraburkholderia* was mixed with *K. pneumoniae* in 50:50 ratio. Only *P. agricolaris* was included in this experiment.

A) Test for effect of competition (*P. agricolaris* alone vs. *P. agricolaris* with *K. pneumoniae*) Model: *P. agricolaris* Population ~ Treatment + *P. agricolaris* strain			
Term	*df*	*F*	*p*
Treatment	1	81.928	1.67×10^{-13}
P. agricolaris strain	4	4.1971	0.004127
Estimated marginal means	Cells/μL	Standard error	
PaNC21 alone treatment	83,066,751	14,398,405	

PaNC21 + *K. pneumoniae* treatment	11,863,058	3,431,678	
Pa159 alone treatment	44,465,278	10,501,895	
Pa159 + *K. pneumoniae* treatment	6,350,244	2,119,519	
Pa161 alone treatment	90,827,413	15,132,375	
Pa161 + *K. pneumoniae* treatment	12,971,386	3,745,515	
Pa31 alone treatment	33,138,894	8,992,156	
Pa31 + *K. pneumoniae* treatment	4,732,683	1,688,341	
Pa317s alone treatment	70,850,490	13,261,176	
Pa317s + *K. pneumoniae* treatment	10,118,410	3,012,969	
B) Test for competition rescue by *D. discoideum* (*P. agricolaris* with *K. pneumoniae* vs. *P. agricolaris* with *K. pneumoniae* & *D. discoideum*) Model: *P. agricolaris* Population ~ Treatment* *P. agricolaris* strain			
Term	*df*	*F*	*p*
Treatment	1	3.7885	0.05614
P. agricolaris strain	4	16.802	2.228×10^{-9}
Treatment* *P. agricolaris* strain	4	46.279	$< 2.2 \times 10^{-16}$
Estimated marginal means	Cells/μL	Standard error	

PaNC21 + *K. pneumoniae* treatment	10,925,271	1,273,457	
PaNC21 + *K. pneumoniae* + *D. discoideum* treatment	42,950,669	2,915,564	
Pa159 + *K. pneumoniae* treatment	5,931,372	1,003,094	
Pa159 + *K. pneumoniae* + *D. discoideum* treatment	7,712,363	1,143,821	
Pa161 + *K. pneumoniae* treatment	14,286,388	1,556,774	
Pa161 + *K. pneumoniae* + *D. discoideum* treatment	5,155,812	935,218	
Pa31 + *K. pneumoniae* treatment	3,882,545	759,148	
Pa31 + *K. pneumoniae* + *D. discoideum* treatment	3,799,310	867,142	
Pa317 + *K. pneumoniae* treatment	11,122,222	1,284,884	
Pa317 + *K. pneumoniae* + *D. discoideum* treatment	375,387	333,828	
Pairwise contrasts	*Z* ratio	*p*	
PaNC21, *K. pneumoniae* vs. *K. pneumoniae* + *D. discoideum* treatment	10.149	< 0.0001	
Pa159, *K. pneumoniae* vs. *K.*	1.167	0.2431	

pneumoniae + *D. discoideum* treatment			
Pa161, *K. pneumoniae* vs. *K. pneumoniae* + *D. discoideum* treatment	-4.816	< 0.0001	
Pa31, *K. pneumoniae* vs. *K. pneumoniae* + *D. discoideum* treatment	-0.072	0.9425	
Pa317, *K. pneumoniae* vs. *K. pneumoniae* + *D. discoideum* treatment	-3.779	0.0002	

Chapter 4: Kin discrimination and competition in bacterial endosymbionts of the social amoeba *Dictyostelium discoideum*, *Paraburkholderia agricolaris* and *P. hayleyella*

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4.1 Abstract

In bacteria kin discrimination, defined as treating non-clonemates differently than clonemates, is common and can involve a variety of mechanisms. However, kin discrimination in intracellular endosymbionts is not much studied. The social amoeba *Dictyostelium discoideum* hosts several bacterial intracellular endosymbionts. We screened two species, *Paraburkholderia agricolaris* and *P. hayleyella* for kin discrimination and found that it was uncommon in both species. The kin discrimination we did find was based on bacteriophage in *P. agricolaris* and a toxin in *P. hayleyella*.

4.2 Introduction

Many eukaryotes interact with bacteria in close relationships called symbioses (Fraune and Bosch 2010; McFall-Ngai et al. 2013). Research on symbiosis has focused on the consequences for the eukaryotic host, but the impact of association on bacterial symbionts is also important (Douglas and Smith 1989; Bronstein 2001; Wilkinson and Sherratt 2001; Kereszt et al. 2011; Garcia and Gerardo 2014). For example, there has been great interest in how social interactions in symbiotic bacteria, particularly pathogens, affect their hosts (Read and Taylor 2001; Bose et al. 2016). Less has been done on how the evolution of symbionts from free-living species affects their interactions with each other as they become more dependent on their hosts.

One class of social interactions common in bacteria is kin discrimination, when individuals treat their genetic relatives differently from non-relatives. An allele that causes its bearer to help others at a cost to itself can evolve if the benefits of helping the recipients exceed the costs to the actor weighed by the probability that they share the allele above background levels in the population, called genetic relatedness (Hamilton 1964a,b). Kin discrimination allows individuals to direct their help to genetic relatives or direct harm to non-kin competitors and benefit kin indirectly. In bacteria kin discrimination can take a variety of forms, such as highly variable narrow-spectrum toxins to which clones are immune (Riley and Wertz 2002; Jamet and Nassif 2015; Hernandez et al. 2020; Ruhe et al. 2020), partially public goods that can only be used by those who share the gene for producing them (Ji et al. 1997; Simms and Bever 1998; Bever and Simms 2000; Denison 2000; Smith et al. 2005; White and Winans 2007; Platt and Bever 2009; Bruce et al. 2017; Stilwell et al. 2018; Aframian and Eldar 2020), and differential adhesion (Trunk et al. 2018; Adams et al. 2019; Ageorges et al. 2019; Nwoko and Okeke 2021). Since bacteria reproduce clonally, kin discrimination often ends up being discrimination between clonemates and non-clonemates (Strassmann et al. 2011; Wall 2016). That said, horizontal gene transfer is common in bacteria and relatedness at the social locus itself may not match average relatedness across the genome (Strassmann et al. 2011).

Kin discrimination is important for bacteria but may not be necessary in an environment with few competitors. While this kind of environment may be rare, an intracellular endosymbiont may experience something like this inside of a host cell. One of the potential benefits of endosymbiosis, when a symbiont lives inside its host, is reduced competition for resources. Intracellular endosymbionts often experience substantial genome degradation over evolutionary time and lose genes that they no longer need (McCutcheon and Moran 2012; Wernegreen 2017;

Husnik and Keeling 2019). Genes for kin discrimination may be among them, provided that endosymbionts are usually clonal, but endosymbioses can also involve co-infection of multiple clones of the same species and when this happens, competition between them can be particularly intense and important for their fitness (Garcia and Gerardo 2014).

The *Dictyostelium discoideum – Paraburkholderia* endosymbiosis is a useful model system in which to test the importance of kin discrimination for endosymbionts that vary in their dependence on their host. *D. discoideum* is a social amoeba that lives in forest soils where it preys on bacteria and reproduces by binary fission. When prey become depleted the social cycle begins wherein individual amoebas begin to aggregate, form a motile slug, then further differentiate into a multicellular fruiting body made up of a ball of spores (sorus) on top of a dead stalk that aids in dispersal to new environments (Kessin 2001; smith et al. 2014). Toxins and bacteria that the amoebas cannot consume are excluded from the sorus by immune-like sentinel cells, which engulf them and fall off the slug as it moves (Chen Guokai et al. 2007; Brock et al. 2016). Despite this mechanism, some environmental bacteria associated with *D. discoideum* can persist through multiple rounds of the social cycle and 42% of wild-collected fruiting bodies contain bacteria of some kind (Brock et al. 2018; Haselkorn et al. 2021).

A quarter of wild-collected isolates of *D. discoideum* contain *P. agricolaris*, *P. hayleyella*, and *P. bonniea* which persistently associate with the amoeba (Haselkorn et al. 2019). These bacteria can infect *D. discoideum* intracellularly, which harms the amoebas by reducing the number of reproductive spores they produce, but also confers them with a “farming” trait that allows them to carry edible food bacteria through the social cycle and seed environments lacking a good food source with their own bacteria (Brock et al. 2011; Shu et al. 2018a; Haselkorn et al. 2019; Khojandi et al. 2019; Brock et al. 2020). These three species represent two independent

transitions to endosymbiosis with *D. discoideum*, one for *P. agricolaris* and one for *P. hayleyella* plus *P. bonniea* which are each other's closest relatives (Brock et al. 2020; Noh et al. 2022).

While all three species can live outside of their hosts, *P. hayleyella* and *P. bonniea* appear to have adapted more to endosymbiosis than *P. agricolaris*. For example, while *P. agricolaris* has a genome size similar to its free-living relatives, *P. hayleyella* and *P. bonniea* have genomes half that size (Brock et al. 2020; Noh et al. 2022). Consistent with the idea that endosymbionts lose genes important for life outside of the host, *P. hayleyella* and *P. bonniea* cannot use many of the carbon sources that *P. agricolaris* and other *Paraburkholderia* are able to use (Brock et al. 2020). The genomes of *P. hayleyella* and *P. bonniea* also have a lower GC content than the genome of *P. agricolaris* (Brock et al. 2020; Noh et al. 2022), which is another hallmark of long-term endosymbiosis (McCutcheon and Moran 2012; Wernegreen 2017; Husnik and Keeling 2019).

P. hayleyella may be more adapted to endosymbiosis with *D. discoideum* than *P. agricolaris* is. For instance, *P. hayleyella* have higher fitness in association with *D. discoideum* than when growing without their host in soil in contrast to *P. agricolaris* which does not benefit in this way (Garcia et al. 2019). In addition, *P. hayleyella* is more harmful than *P. agricolaris* to naturally bacteria-free *D. discoideum*. However, *P. hayleyealla* also harms the *D. discoideum* it was isolated from less than *P. agricolaris* harms its own natural host strain (Shu et al. 2018a). *P. hayleyella* infects more *D. discoideum* spores than *P. agricolaris*, but spores have more *P. agricolaris* bacteria within them (Shu et al. 2018a; Miller et al. 2020). Ultimately *P. hayleyella* ends up with larger population sizes than *P. agricolaris* when associated with *D. discoideum* because it is both more infectious and does less damage to the host (Miller et al. 2020). Both *P. agricolaris* and *P. hayleyella* swim towards conditioned media prepared from *D. discoideum*

more than other *Paraburkholderia* species do (Shu et al. 2018b). Interestingly, *P. hayleyella* prefers conditioned media of *D. discoideum* that have already been infected by *P. hayleyella* or *P. agricolaris* while the latter does not have this preference (Shu et al. 2018b). This suggests a context where kin discrimination could be important, at least for *P. hayleyella*.

We screened 10 strains of *P. agricolaris* and 10 strains of *P. hayleyella* for kin discrimination, expecting that it would be common in these species because it is common in bacteria in general. We hypothesized that kin discrimination should be stronger or more common in *P. agricolaris* than in *P. hayleyella* because the former relies on its host for fitness less than the latter. Therefore, *P. hayleyella* may not need kin discrimination as much as a species that may spend more time in the environment away from its host. We used three assays to screen for kin discrimination, a swimming assay, an agar diffusion assay, and a mixed culture assay.

For our swimming assay we inoculated soft agar with clones of *P. agricolaris* or *P. hayleyella* and looked for the formation of boundaries between different clones as they swam outwards.

During swarming, a kind of social motility over surfaces, incompatible strains form visible boundaries between swarms while isogenic swarms merge. This has been observed in *Proteus mirabilis* (Dienes 1946; Senior 1977; Budding et al. 2009), *Bacillus subtilis* (Stefanic et al. 2015), *Pseudomonas aeruginosa* (Munson et al. 2002) and *Myxococcus xanthus* (Vos and Velicer 2009). These phenotypes are due to killing or inhibition by toxins in *P. mirabilis* (Kusek and Herman 1980; Cardarelli et al. 2015), *B. subtilis* (Lyons et al. 2016), and *M. xanthus* (Dey et al. 2016). While *P. agricolaris* and *P. hayleyella* do not swarm over surfaces, they can swim through liquids.

Many bacteria have bacteriocins which are toxins that are only effective against other clones of the same species or closely related species (Riley and Wertz 2002; Ruhe et al. 2020). We used an agar diffusion assay to test if clones of *P. agricolaris* or *P. hayleyella* produced toxins that were effective against other clones.

Finally, we co-cultured different clone pairs together of either *P. agricolaris* or *P. hayleyella* and measured their growth rate, carrying capacity and lag time. For each measure, we compared the average of two clones cultured independently to that measure for the two clones cultured together. We did this in order to test if on average two different clones cultured together have a different, for example, growth rate than they would if the growth rates of each clone were averaged.

4.2 Materials and methods

4.2.1 Culture conditions

For each experiment we grew *Paraburkolderia* clones from 20% glycerol freezer stocks by streaking out bacteria on YG (3g agar, 5g yeast extract, 4g glucose, 1g NaCl per liter) agar plates and incubating for 3-4 days at room temperature. We collected bacteria, diluted in KK2 [2.25 g KH2PO4 and 0.67 g K 2HPO4 per liter] buffer to 1.5 OD_{600} and kept stocks at 4°C until use (1 day).

4.2.2 Swimming assay

We used sterile toothpicks to inoculate 30ml of soft (0.3%) YG agar in petri dishes with pairs of clones of either *P. agricolaris* (n=10) or *P. hayleyella* (n=10) 1cm apart in duplicate. We incubated petri dishes at room temperature for three days, then photographed them and visually

determined if there was a boundary between motility halos. There were no further differences after seven days of growth.

4.2.3 Agar diffusion assay

For each clone of *P. agricolaris* and *P. hayleyella* we infused 3ml of 0.5% water agar with 200µl of bacteria diluted to 1.5 OD_{600} in KK2 buffer, poured it in a thin layer over 30 ml of solidified YG agar in petri dishes, and allowed the water agar to cool and solidify. We call these the response clones. We then spotted 2µl of each of 10 clones (test clones) of the same species on top of the water agar in triplicate and incubated them at room temperature for three days. We then looked for the presence of zones of inhibition of the response clones around the spotted test bacteria. We repeated this experiment three times for each species.

4.2.3 Bacteriophage testing

We grew 200µl of bacteria in 3ml of liquid YG media at 30°C shaking and 225 rpm for twenty hours for each of the following clone combinations that showed the possible presence of bacteriophage in the agar diffusion assay: Pa70 alone, Pa70 and Pa317s, Pa70 and Pa317t, Pa70 and Pa80, Pa161 alone, Pa161 and Pa317s, Pa161 and Pa317t, and Pa161 and Pa80. We then pelleted bacteria by centrifuging 1ml of the liquid culture at 10,000 x g for 10 minutes and filtered the supernatant through 0.22 µm syringe filters to remove any remaining bacteria. We spotted 2µl of the filtrate on 0.5% water agar infused with 200 µl of each clone of *P. agricolaris* diluted to 1.5 OD_{600} on top of 30ml of YG agar. We also plated 200µl of each filtrate on 30ml of YG agar to check for bacterial growth. We checked for the presence of phage plaques, clearings in the agar made by lytic phage, after one day.

4.2.4 Mixed culture assay

In order to screen for kin discrimination not detected in the previous assays, we made mixed cultures of each clone pair (0.5% by volume of each clone) or monocultures (1% by volume) in 96-well plates in 200ul of liquid YG media. We incubated the cultures in a Tecan microplate reader at room temperature and measured OD_{600} every hour for 72 hours. We had 1-2 technical replicates per plate and repeated the experiment three times. We measured growth rate, carrying capacity and lag time for mixed culture pairs and monocultures of 10 *P. agricolaris* clones and 10 *P. hayleyella* clones in order to determine if these clones interacted with each other. We measured growth rate using the package fitr (Angst 2022) in R (version 4.2.1, R Foundation for Statistical Computing) with the default parameters. We defined carrying capacity as the maximum OD_{600} reached during the growth period. We defined lag time as the time until the maximum growth rate calculated with fitr was reached. If the mixed culture of two clones grew no differently from the average of the two clones growing independently then we consider this a lack of kin discrimination.

4.2.5 Statistical analyses

For the mixed culture assay we used paired Wilcoxon signed rank tests to determine whether the growth rate of pairs of clones was significantly different from the mean of the individual intrinsic growth rates of each of those clones separately. We repeated this with carrying capacity and lag time. All analyses were done in R (version 4.2.1, R Foundation for Statistical Computing).

4.3 Results

4.3.1 Swimming assay shows uniform boundary formation in *P. agricolaris* and *P. hayleyella*

We found that motility halos formed by swimming bacteria uniformly flattened into boundaries regardless of whether they were paired with their clonemates or not (Figs. 2 and 3, Table 1). We

also found that one clone of *P. agricolaris* (Pasoil99) and one clone of *P. hayleyella* (Ph69) did not form motility halos under these conditions and so could not be scored for the presence or absence of boundaries.

4.3.2 Agar diffusion assay shows presence of bacteriophage in *P. agricolaris* and a diffusible toxin in *P. hayleyella*

We screened for the presence of diffusible toxins by using a modified agar diffusion assay (Hoover and Harlander 1993). We found production of a diffusible toxin by only one test clone of one species, Ph69, which was effective against all other *P. hayleyella* clones (Fig. 4, Table 1). In *P. agricolaris*, we found that two test clones (Pa70 and Pa161) cause apparent phage plaques, clearings where lytic bacteriophage have killed bacteria, around them when spotted on three response clones (Pa317t, Pa317s, and Pa80) (Fig. 5, Table 1). We investigated these further.

4.3.3 Bacteriophage testing shows bacteria-free filtrate forms phage plaques on sensitive clones

To exclude a direct effect of bacteria in causing the phage plaque–like clearings, we collected and tested bacteria-free filtrates. Bacteria-free filtrate from liquid cultures containing Pa70 or Pa161 formed phage plaques on Pa317t, Pa317s and Pa80 and induction of bacteriophage production did not require the presence of test clones. In fact, there were fewer plaques when Pa70 or Pa161 were co-cultured with clones sensitive to the bacteriophage. There were no phage plaques observed when filtrate from these cultures were spotted on any other *P. agricolaris* clones. Though the same volume and concentration of filtrate was spotted one each plate, there

were more phage plaques on Pa317t and Pa317s than on Pa80 (Fig. 6). We observed no bacterial growth on the negative control plates, meaning that there was no *P. agricolaris* in the filtrate.

4.3.4 Mixed cultures showed some evidence of better growth, contrary to kin discrimination expectations

Under kin discrimination we may expect mixed cultures to do worse: having a lower growth rate or carrying capacity, or a longer lag time. For *P. agricolaris*, we found that the growth rate of mixed cultures was not significantly different from the mean of the growth rates of the two clones independently (Wilcoxon signed rank test, V = 13909, p-value = 0.3277) (Fig. 7). This indicates that the growth rate of mixed cultures did not differ from our null expectation of no interaction between the clones. We also found that the carrying capacity of the mixed cultures was significantly greater than the mean of the carrying capacities of the two clones independently (Wilcoxon signed rank test, V = 4222.5, p-value = 2.2e-16) (Fig. 8). This means that the mixed culture pairs had higher carrying capacities than the individual clones did separately. This could be because of facilitation between the clones, such as cross feeding, or resource partitioning. Finally, we found that the lag time of mixed cultures was significantly shorter than the mean of the lag times of the two clones independently (Wilcoxon signed rank test, V = 12245, p-value = 0.01113) (Fig. 9). This means that mixed cultures had shorter lag times than expected from the two clones growing independently. This could be consistent with either facilitation or competition. It may be beneficial to have a shorter lag time in order to outcompete other clones, or it could just be the result of facilitation.

We separately analyzed these data for the 6 pairs that showed the presence of phage in the agar diffusion assay and the results were quite different. We found that these pairs had significantly lower growth rates in mixed culture than on their own (Wilcoxon signed rank test, V = 567, p-value = 1.726e-07), did not have significantly different carrying capacities in mixed culture compared to the mean of their independent carrying capacities (Wilcoxon signed rank test V = 211, p-value = 0.143), and had significantly longer lag times in pairs than on their own (Wilcoxon signed rank test V = 85, p-value = 0.0002857) (Figs. 1a and 1b). These are consistent with our expectations for kin discrimination.

For *P. hayleyella*, we found that the growth rate of mixed cultures was significantly higher than the mean of the growth rates of the two clones independently (Wilcoxon signed rank test, V = 7314, p-value = 5.445e-10) (Fig. 7). This means that the mixed cultures grew faster than expected from the growth rates of the two clones in the pair independently. This could be due to either cross-feeding or reduction of competition. We also found that the carrying capacity of the mixed cultures was not significantly different from the mean of the carrying capacities of the two clones independently (Wilcoxon signed rank test, V = 12260, p-value = 0.1513) (Fig. 8). Finally, we found that the lag time of mixed cultures was significantly shorter than the mean of the lag times of the two clones independently (Wilcoxon signed rank test, V = 12805, p-value = 0.0007561) (Fig. 9). This could again be due to either facilitation or competition.

4.4 Discussion

We used three assays to try to detect kin discrimination in the endosymbionts *P. agricolaris* and *P. hayleyella*. We initially noticed boundaries forming between clones in these bacteria similar to those reported in other bacteria and investigated further. We first used a phenotypic assay to

determine if boundaries formed between clones or if they merged seamlessly, which we called the swimming assay.

We found that each clone formed a boundary between itself and the clone it was paired with, even if they were the same clone (Figs. 2 and 3). This uniform boundary formation across clones is similar to behavior in *Paenibacillus dendritiformis* and *Bacillus subtilis hag* mutants, which likewise avoid other colonies of the same genotype (Be'Er et al. 2009; James et al. 2009; Be'er et al. 2010). This could be a way of generally avoiding intraspecific competition or could be due to bacteria simply avoiding areas that have already been depleted of resources (Strassmann et al. 2011). This is not kin discrimination because clones do not treat non-kin differently from kin.

Our second assay was a screen for toxins effective against other clones of the same species (Figs. 4 and 5). These are widespread in bacteria (Riley and Wertz 2002; Ruhe et al. 2020) so we initially expected that we might find many clones that produced toxins and many that were susceptible to them. We first found that one clone of *P. hayleyella*, Ph69, produced a toxin or toxins that was effective against every other clone of *P. hayleyella* that we tested. This is kin discrimination because non-kin are treated differently than kin. However, that does not necessarily mean that this toxin or toxins evolved because of selection for kin discrimination. It may be that it evolved as a general antibiotic effective against many bacteria or other species that happens to also be effective against other *P. hayleyella*.

Second, we found that two clones of *P. agricolaris*, Pa70 and Pa161, produce a bacteriophage (or perhaps two different bacteriophages with the same host range) that are effective against three of the eight other clones: Pa317t, Pa317s, and Pa80. Here, there was not a large zone of inhibition where bacteria could not grow like there was with the toxin in Ph69. Instead, there was

a series of small clearings merging together around the outside of the colonies, which could indicate the presence of bacteriophage. We grew Pa70 and Pa161 in liquid culture, filtered out the bacteria and spotted the filtrate on Pa317s, Pa317t, and Pa80 to look for signs of bacteriophage. We found that the filtrate made small clearings and that the number of clearings were reduced as the filtrate was diluted (Fig. 6). This is indicative of bacteriophage rather than a diffusible toxin. Toxins make single clearings that become fainter as dilution increases while bacteriophage make small distinct clearings that decrease in number as the dilution increases because each clearing originates from a single viral particle. This bacteriophage can be considered kin discrimination because non-kin are susceptible to the bacteriophage while kin are not. But, again, it would be difficult to argue that it evolved in order to provide kin discrimination. It seems more likely that kin discrimination is a side effect of host-phage interactions, with some host clones being resistant to the phage and therefore able to carry it, and other clones not being resistant.

There are a few other examples of phage involved in kin discrimination between bacteria. In *M. xanthus*, non-motile cells prevent non-kin from swarming by using toxins derived from a prophage (Dey et al. 2016). In *E. coli*, bacteriophage can cause boundaries to form between motility halos of competing clones similar to the ones we found in our swimming assay (Song et al. 2019). However, in *P. agricolaris*, all clone pairs that made motility halos also made boundaries between them so the bacteriophage are probably not involved in this phenotype. Kin discrimination via bacteriophages in this species may only be important in environmental contexts where mixing with other clones is unavoidable.

Our third assay was to co-culture clones in liquid culture in a microplate reader and measure their population size over time. From these data we calculated maximum growth rate, carrying

capacity and lag time. We compared the growth rate of mixed cultures to the mean of the growth rate of each clone cultured separately, with the null hypothesis that they were not different. We repeated this kind of test for carrying capacity and lag time for each species. This assay was intended to pick up on kin discrimination not detected by our other two assays, where the antagonism of kin discrimination might lead to lower growth parameters. We found that when in mixed culture *P. agricolaris* had significantly greater carrying capacities than expected from the average of the individual clones, significantly shorter lag times than expected, and the same growth rates as expected. We also found that *P. hayleyella* had significantly higher growth rates than expected when in mixed culture and well as significantly shorter lag times, but not significantly different carrying capacities. This could be evidence for facilitation between the clones within each species because mixed cultures on average grow better than their constituents grow on their own. This is probably due to cross feeding or resource partitioning rather than kin discrimination because the effects are in the opposite direction than we would expect if there was kin discrimination. It is surprising because resource partitioning is usually between species rather than within species, but could be due to genes lost in *P. hayleyella* and not *P. agricolaris*.

Overall, we found that kin discrimination is rare between clones of *P. agricolaris* or *P. hayleyella*. This could be because both species are endosymbionts of *D. discoideum* and so have lost genes important for kin discrimination. Alternatively, it could be that kin discrimination is uncommon in their ancestors despite being common among bacteria in general. Work on other species of *Paraburkholderia* could resolve this question. Finally, kin discrimination may not be important for these species if relatedness is high in nature, which could be due to passive processes like population structure or the result of the general avoidance behavior we found in the swimming assay.

4.5 References

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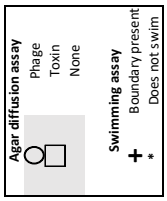
4.6 Figures



Paraburkholderia agricolaris

Clone infused in overlay	Clone ID	Clone spotted on overlay										
		31	1045	nc21	159	80	317s	317t	70	161	soil99*
	31	+	+	+	+	+	+	+	+	+	+
	1045	+	+	+	+	+	+	+	+	+	+
	nc21	+	+	+	+	+	+	+	+	+	+
	159	+	+	+	+	+	+	+	+	+	+
	80	+	+	+	+	+	+	+	+	+	+
	317s	+	+	+	+	+	+	+	+	+	+
	317t	+	+	+	+	+	+	+	+	+	+
	70	+	+	+	+	+	+	+	+	+	+
	161	+	+	+	+	+	+	+	+	+	+
	soil99*	+	+	+	+	+	+	+	+	+	+

Paraburkholderia hayleyella

Clone infused in overlay	Clone ID	Clone spotted on overlay									
		11	21	22	23	38	45	529	155	410	69*
	11	+	+	+	+	+	+	+	+	+	+
	21	+	+	+	+	+	+	+	+	+	+
	22	+	+	+	+	+	+	+	+	+	+
	23	+	+	+	+	+	+	+	+	+	+
	38	+	+	+	+	+	+	+	+	+	+
	45	+	+	+	+	+	+	+	+	+	+
	529	+	+	+	+	+	+	+	+	+	+
	155	+	+	+	+	+	+	+	+	+	+
	410	+	+	+	+	+	+	+	+	+	+
	69*	+	+	+	+	+	+	+	+	+	+

Table 4.1. Results of assays of kin discrimination in *Paraburkholderia agricolaris* (top) and *P. hayleyella* (bottom). Two clones, Pasoil99 and Ph69, did not swim (Figs. 4.2 and 4.3) and so could not be scored for boundary formation. All other clone pairs formed boundaries between motility halos, including self-self pairs. Two clones of *P. agricolaris*, Pa70 and Pa161, formed

phage plaques (small clearings made by bacteriophage) around their colonies when spotted on three other clones, Pa80, Pa317s, and Pa317t (Figs 4.5 and 4.6). One clone of *P. hayleyella*, Ph69, formed a zone of inhibition (clearing) around its colonies when spotted on each other clone (Fig 4.4).

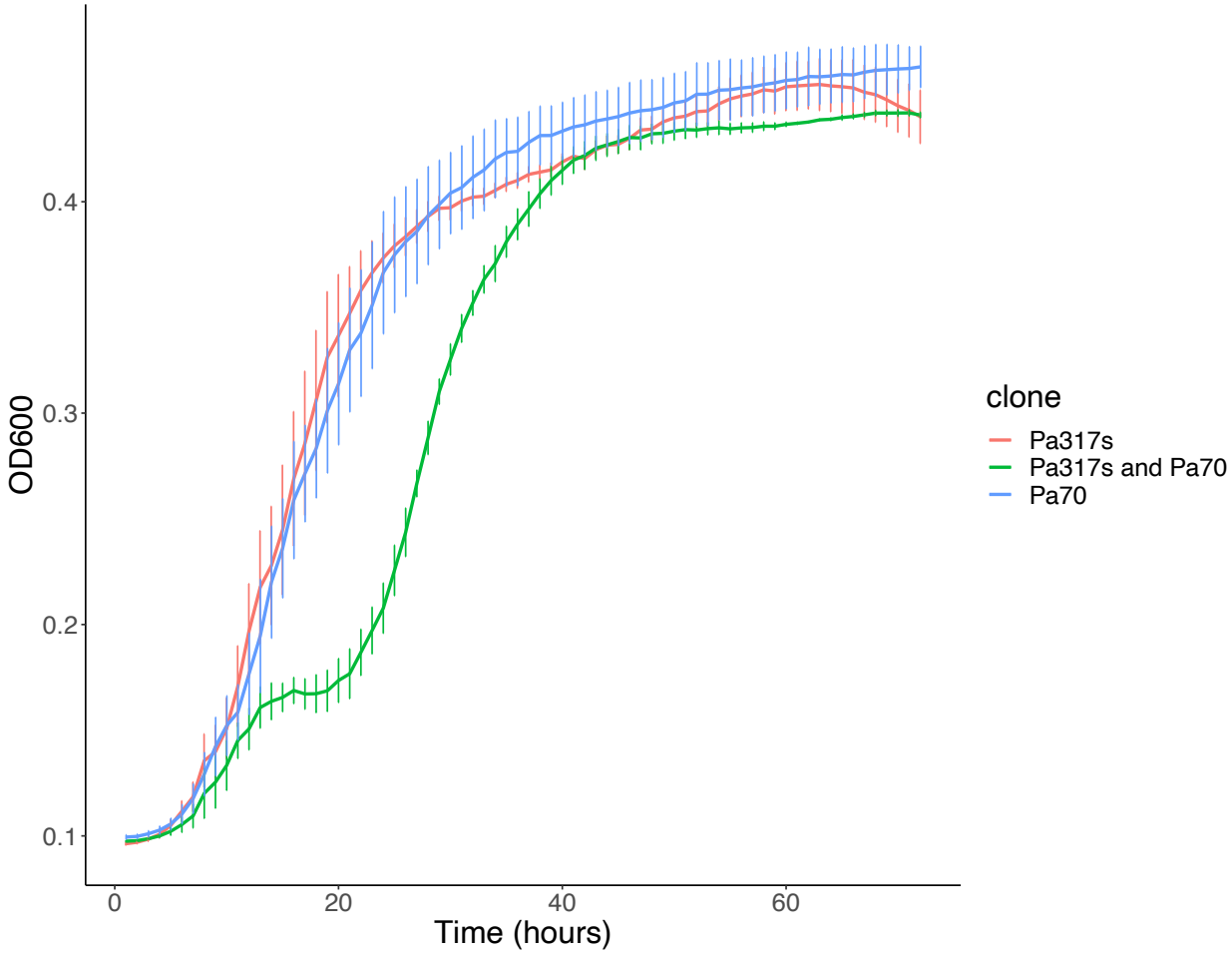


Figure 4.1a. Example growth curves of two clones grown alone and in mixed culture which showed the presence of phage in the agar diffusion assay (Figs. 4.5 and 4.6). The mixed culture growth curve shows a delayed lag time compared to either of the clones on their own.

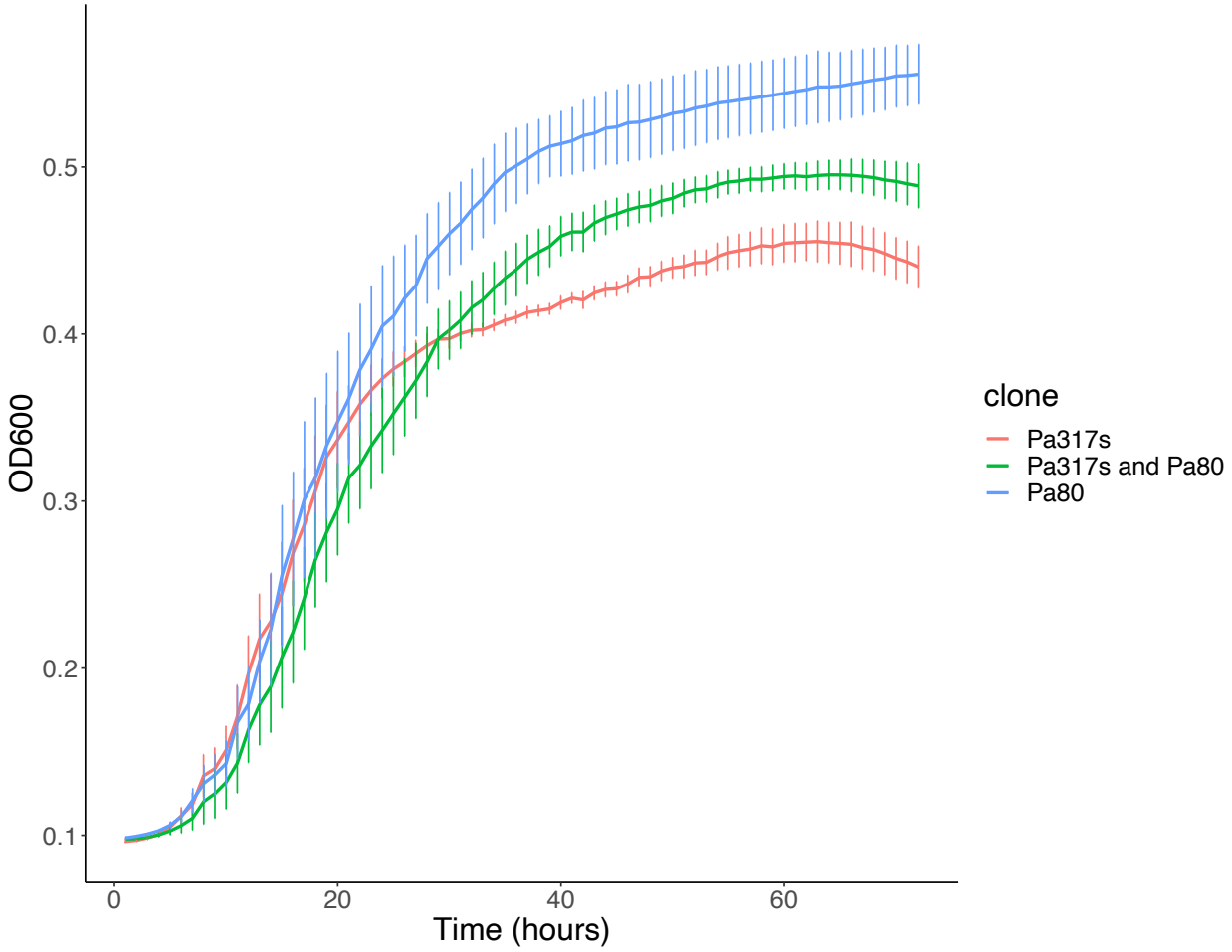


Figure 4.1b. Example growth curves of two clones grown alone and in mixed culture which did not show the presence of phage in the agar diffusion assay (Figs. 4.5 and 4.6). The mixed culture does not show the lag described in Fig. 4.1a.

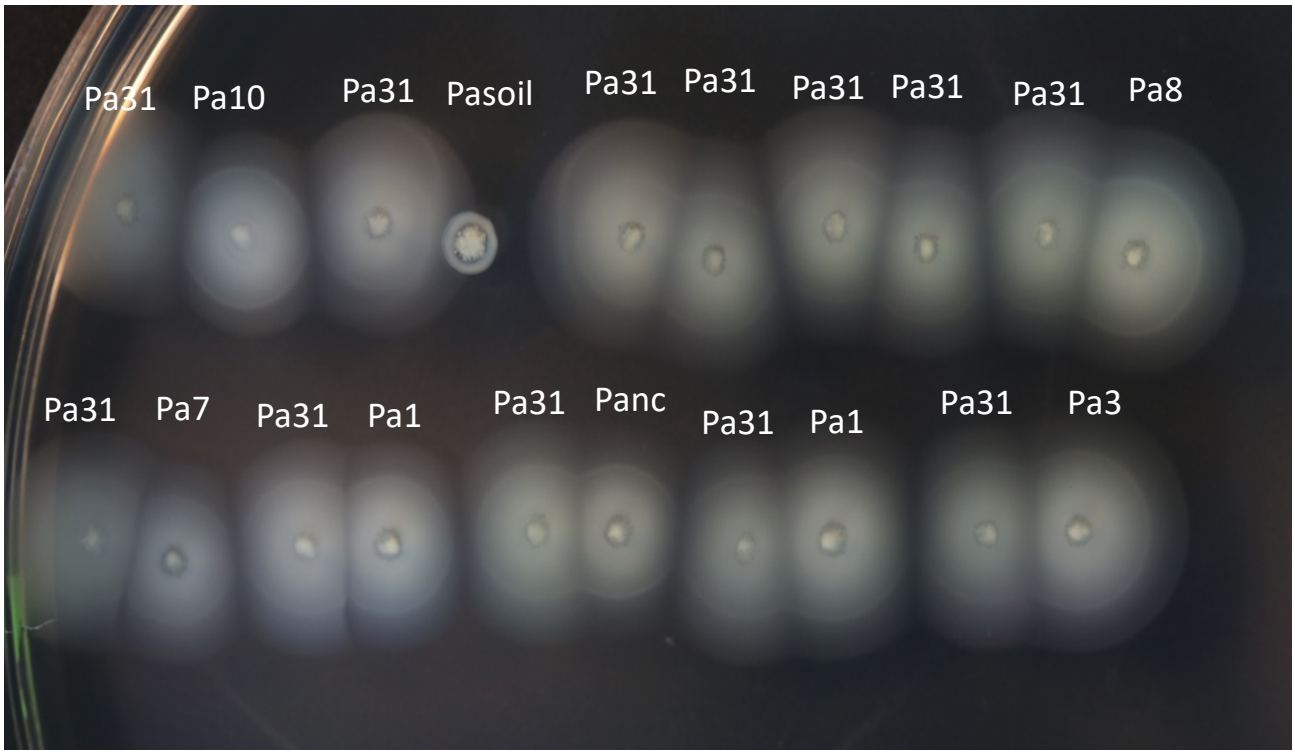


Figure 4.2. Example swimming assay for *P. agricolaris*. Boundaries form between all colony pairs, regardless of whether they were clonemates or non-clonemates. Pasoil99 does not swim.

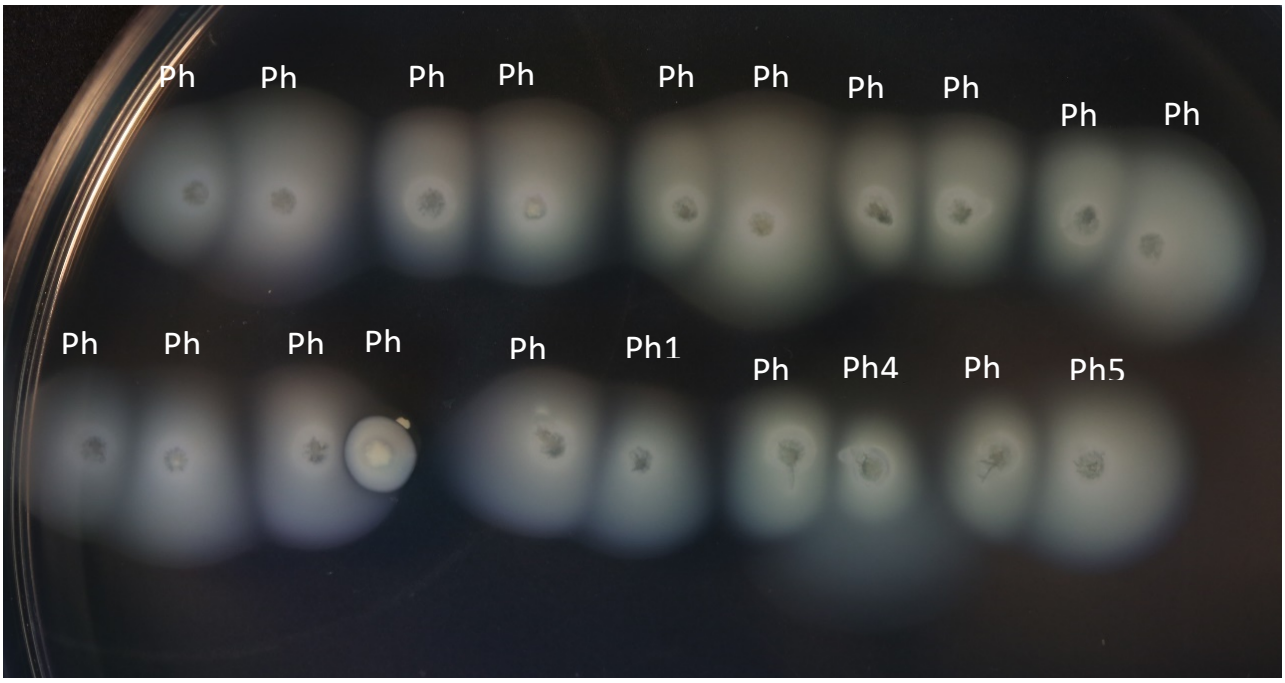


Figure 4.3. Example swimming assay for *P. hayleyella*. Boundaries form between all colony pairs, regardless of whether they were clonemates or non-clonemates. Ph69 does not swim.

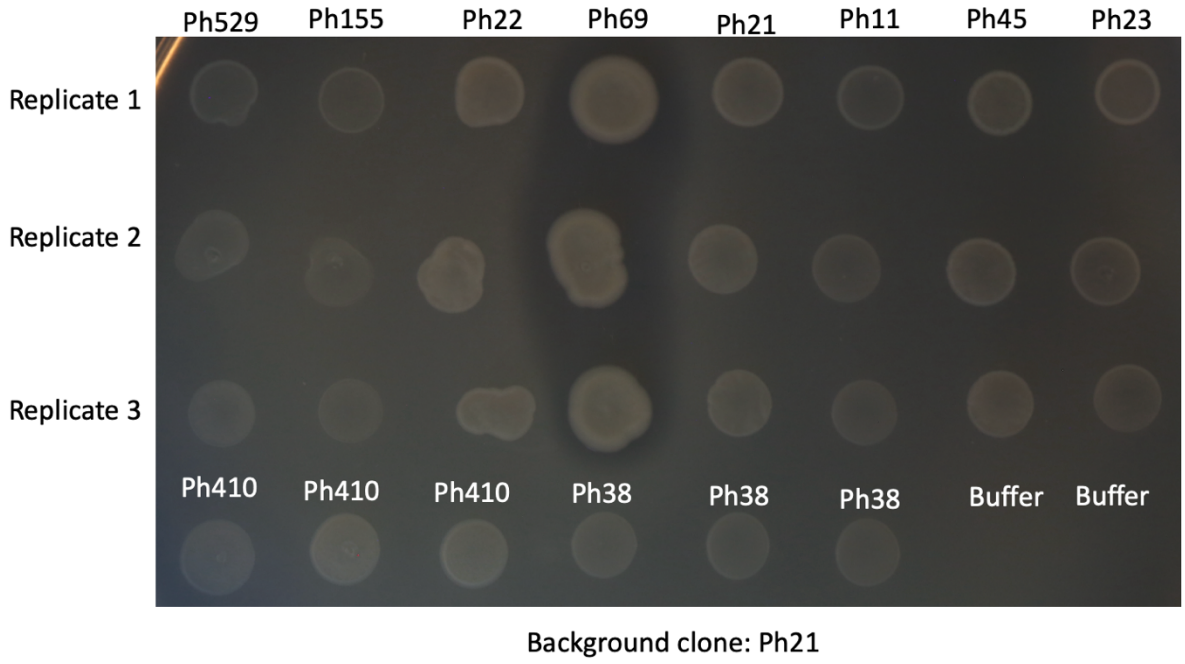


Figure 4.4. Example agar diffusion assay for *P. hayleyella*. Ph21 is infused in a thin layer of agar then other clones are spotted on top. The dark area around Ph69 is a bacteria-free clearing which indicates the presence of a diffusible toxin. Eight test clones are shown in triplicate in the first three rows. The last row includes the other test clones and buffer controls.

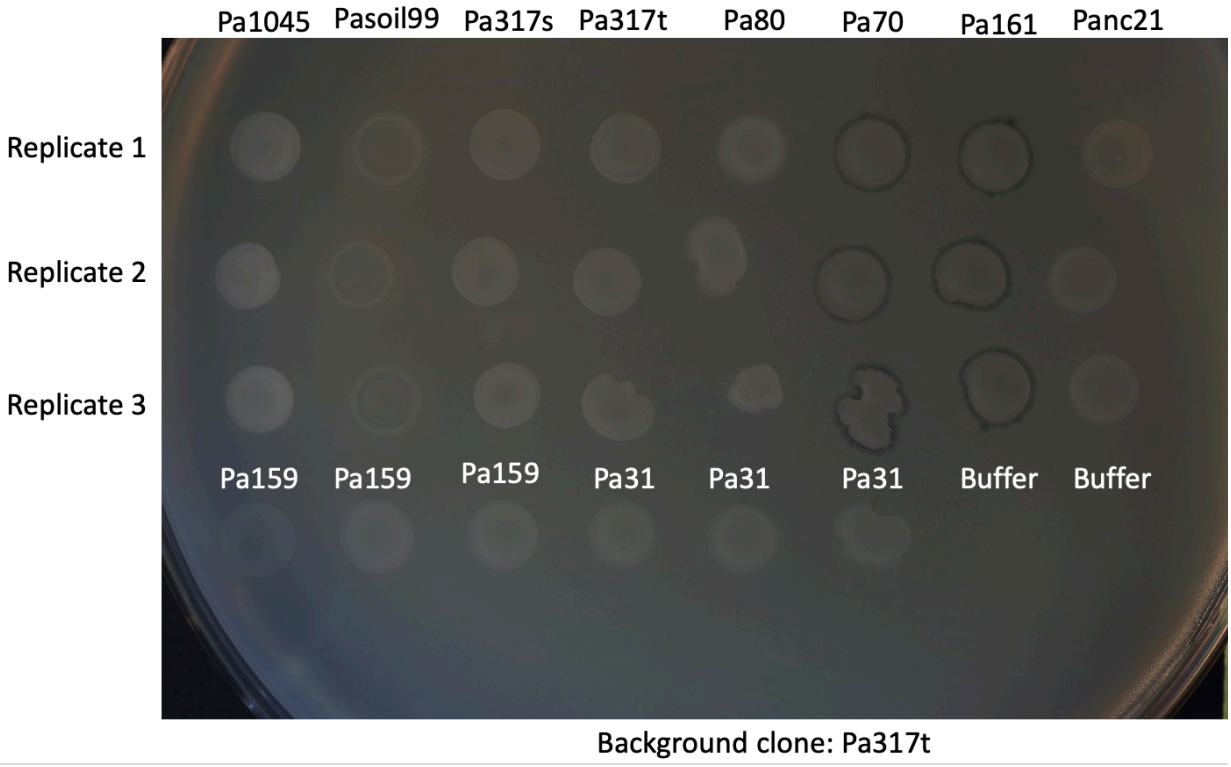


Figure 4.5. Example agar diffusion assay for *P. agricolaris*. Pa317t is infused in a thin layer of agar then other clones are spotted on top. The dark areas around Pa70 and Pa161 are made up of many small bacteria-free clearings which suggests the presence of a bacteriophage. Eight test clones are shown in triplicate in the first three rows. The last row includes the other test clones and buffer controls.

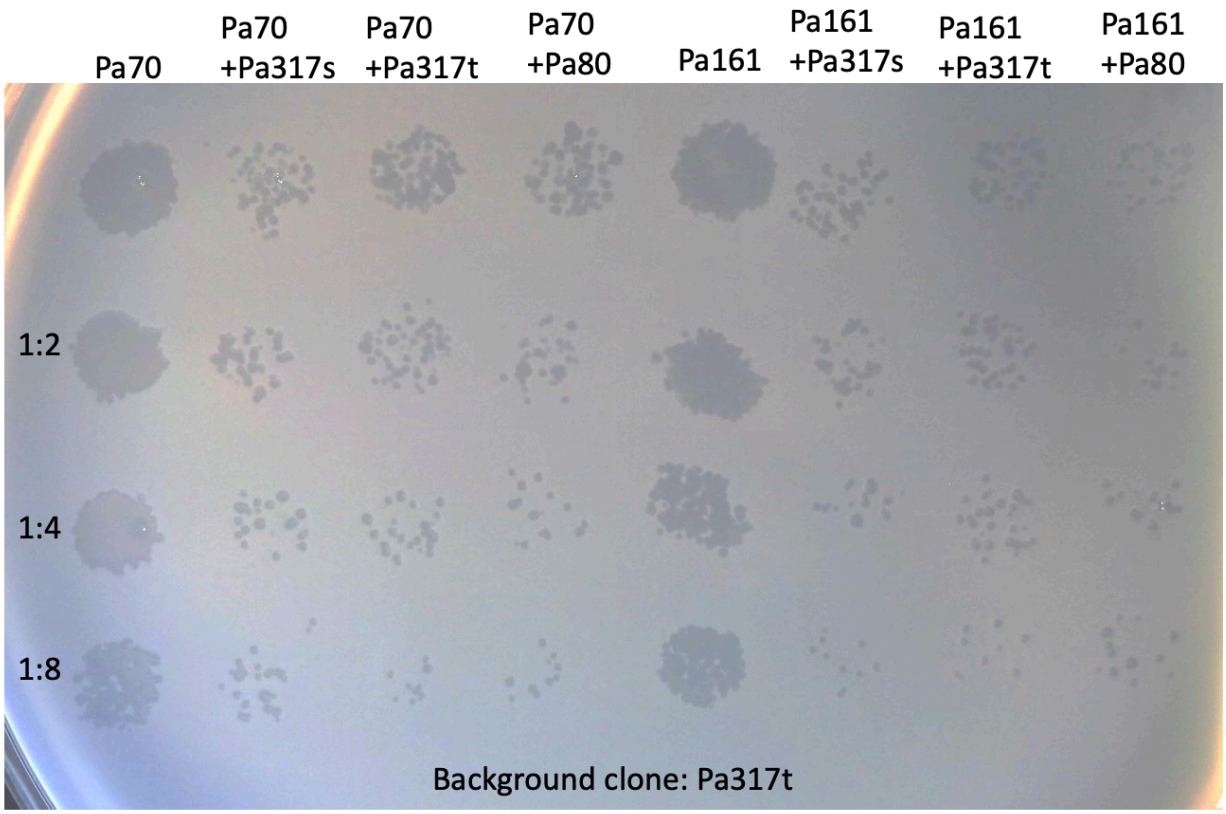


Figure 4.6. Bacteriophage assay. Pa317t is infused in a thin layer of agar as in Figure 5, then bacteria-free filtrates are spotted on top. Each column is a liquid culture containing the indicated clones and each row is a dilution of that filtrate with dilution levels indicated on the left of the image. The dark spots are small clearings in the agar, indicating the presence of bacteriophage.

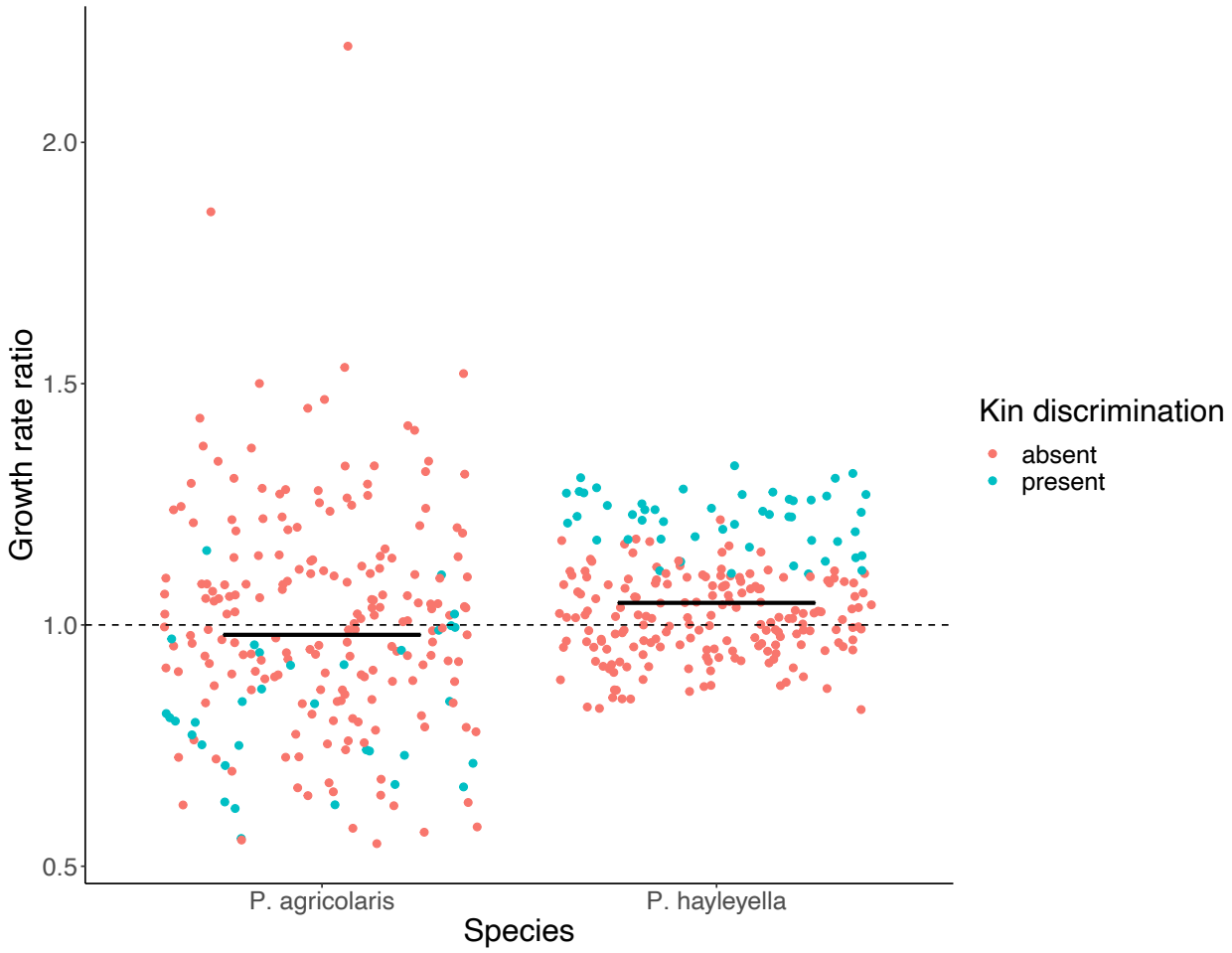


Figure 4.7. Growth rate of mixed cultures divided by the mean of each clone in the pair cultured individually. Kin discrimination refers to the presence or absence of bacteriophage in *P. agricolaris* pairs and the presence or absence of a toxin in *P. hayleyella* pairs. The dashed line represents the null hypothesis of no interactions between the clones. The black bars represent the median.

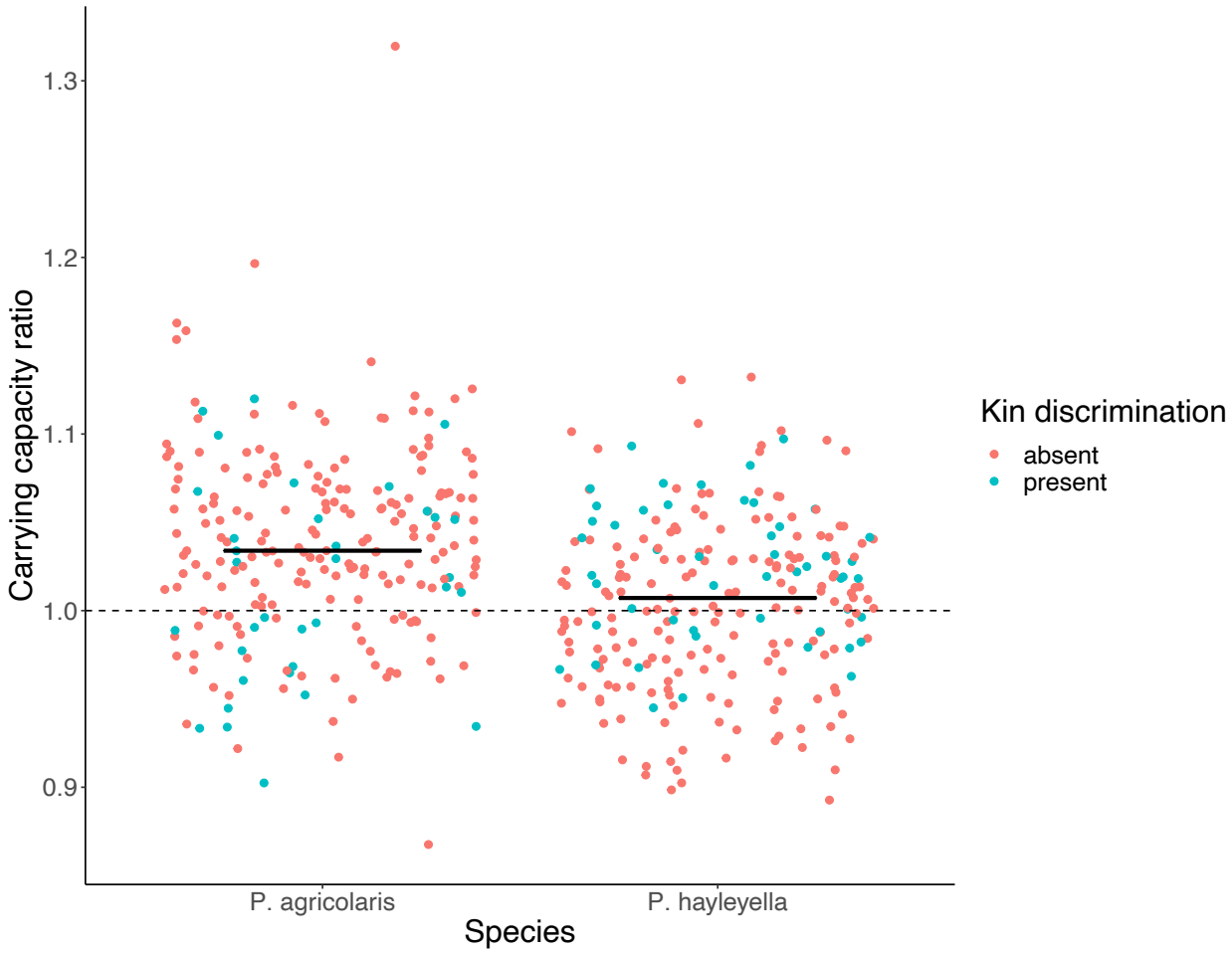


Figure 4.8. Carrying capacity of mixed cultures divided by the mean of each clone in the pair cultured individually. Kin discrimination refers to the presence or absence of bacteriophage in *P. agricolaris* pairs and the presence or absence of a toxin in *P. hayleyella* pairs. The dashed line represents the null hypothesis of no interactions between the clones. The black bars represent the median.

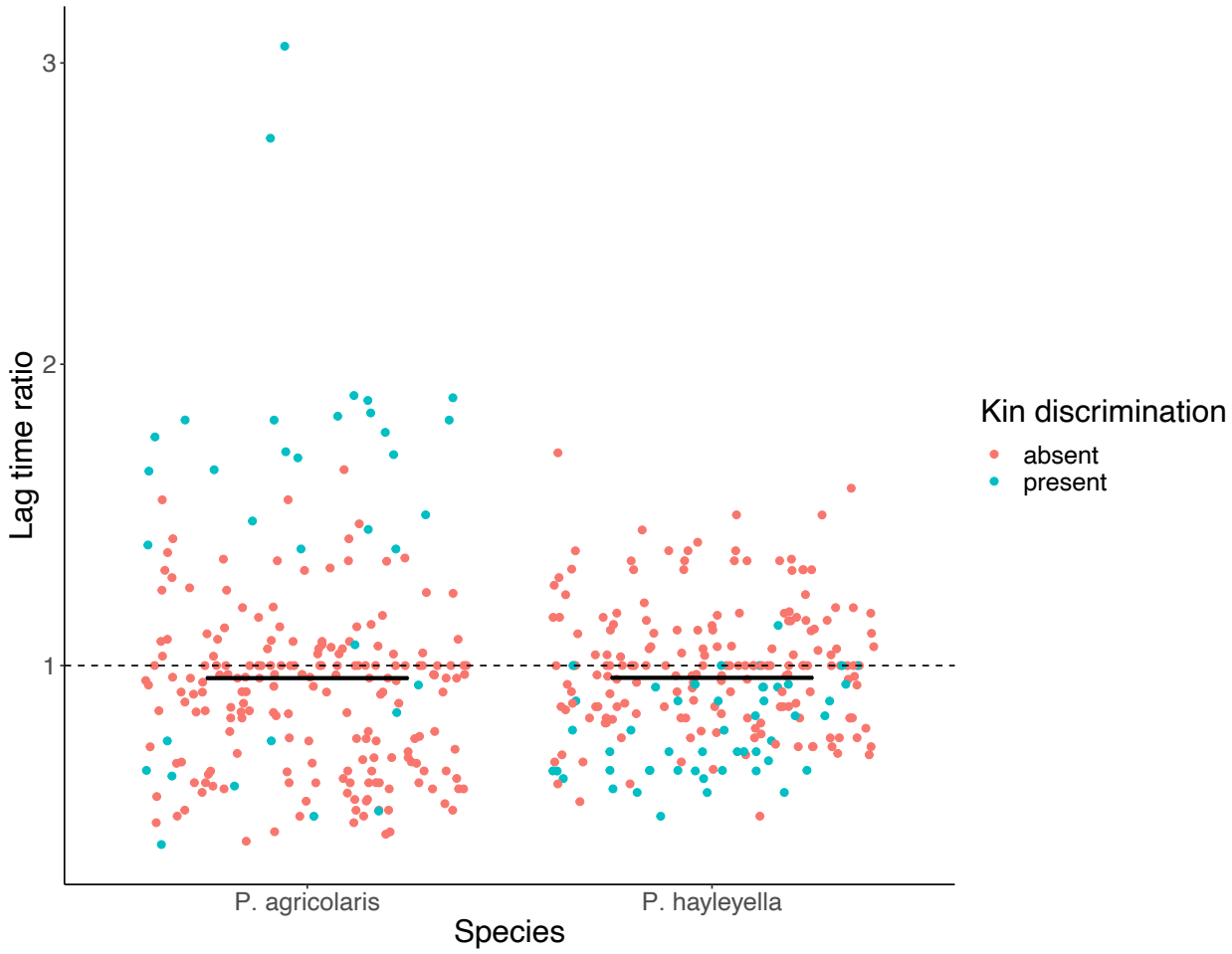


Figure 4.9. Lag time of mixed cultures divided by the mean of each clone in the pair cultured individually. Kin discrimination refers to the presence or absence of bacteriophage in *P. agricolaris* pairs and the presence or absence of a toxin in *P. hayleyella* pairs. The dashed line represents the null hypothesis of no interactions between the clones. The black bars represent the median.