Paraburkholderia bonniea symbiont strain identity impacts amoeba host fitness and rate of horizontal transmission

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Abstract (250)

Whether coevolution determines distribution patterns of facultative symbionts among individuals of a host species is an open question. We used the *Dictyostelium discoideum-Paraburkholderia* system to investigate the role of host fitness and symbiont transmission in co-occurrence patterns of naturally occurring (native) host-symbiont strain pairs. *P. bonniea* is able to persistently infect *D. discoideum* in the lab, yet few wild *D. discoideum* isolates natively host this symbiont. If native host-symbiont combinations lead to higher fitness for host and symbiont compared to nonnative combinations, we assumed a stronger chance that coevolution had led to their co-occurrence. We approached the factors that influence the fitness consequences of host-symbiont interactions in terms of host defense mechanisms and symbiont virulence mechanisms. Given a specific combination of host and symbiont, variable host resistance and symbiont proliferation lead to variation in symbiont density, while variable host tolerance and symbiont benevolence lead to variation in host fitness relative to symbiont density. We used both vegetative and social stages of the host life cycle to approximate interactions between *D. discoideum* and *P. bonniea*, when hosts form social groups and symbiont horizontal transmission is facilitated by these social groups. We found no difference in host defense mechanisms between native and nonnative hosts of *P. bonniea*, but *P. bonniea* possesses variable phenotypes in proliferation and benevolence. More importantly, variation in symbiont benevolence led to increased horizontal transmission of benevolent strains compared to malevolent strains during the social stage of the host life cycle.

Importance (150)

Hosts and symbionts in a facultative symbiosis do not depend on each other for survival. Perhaps due to this labile relationship, we lack a solid understanding of why only some individual hosts carry facultative symbionts rather than all. We used the social amoeba *Dictyostelium discoideum* and its bacterial symbiont *Paraburkholderia bonniea* to test whether naturally occurring (native) host-symbiont strain pairs are the result of coevolution. We find that native combinations of hosts and symbionts do not enjoy improved fitness compared to nonnative combinations. Instead, both host and symbiont fitness depend on symbiont identity, as *P. bonniea* strains varied in their ability to proliferate within groups of hosts and in the degree

of fitness loss they caused given a similar infection prevalence. More importantly, variation in the latter property, known as benevolence, led to increased horizontal transmission of benevolent strains compared to malevolent strains during the social stage of the host life cycle.

Running title

Symbiont benevolence impacts horizontal transmission

Introduction

Symbiosis can be broadly defined as an intimate and prolonged association among different organisms, inclusive of positive, neutral, or negative fitness outcomes to the partners involved. Host-symbiont relationships can be obligate or facultative. Obligate symbioses, when host and symbiont need each other to survive, are expected to be the result of reciprocal selection between host and symbiont over a long period of evolutionary time (Frank 1995; Law and Dieckmann 1998). In contrast, facultative symbioses, in which host and symbiont can each survive in a free-living state, are often assumed to be the result of shorter term relationships (Moran et al. 2008). However, stable long-term facultative symbiotic relationships may be more common than expected. For example, phylogenetic codivergence patterns support the stability of the ancient facultative symbiosis between *Glomeribacter* and Glomeromycota fungi (Mondo et al. 2012). Recurring co-infection patterns of specific symbionts support the stability of heritable facultative microbial communities that infect the pea aphid *Acyrthosiphon pisum* (Rock et al. 2018). Stable facultative symbioses are well suited to reveal how symbiotic relationships are initiated and maintained over evolutionary time, because both host and symbiont can independently evolve in response to any changes in the ecological context of the symbiotic relationship. However, many aspects of facultative symbioses are relatively poorly understood by virtue of the labile relationship. This includes which factors determine distribution patterns of facultative symbionts among individuals within a host species (Niepoth et al. 2018). For example, we lack a solid understanding of why some individual hosts carry facultative symbionts while others do not.

The potential for, and outcome of, long-term association between a host and any potential symbionts is determined by host genetics, symbiont genetics, and various environmental factors (Schmid-Hempel 2011). We approached the specific factors that influence the fitness consequences of host-symbiont interactions in terms of host defense mechanisms and symbiont virulence mechanisms. Host resistance (the ability to limit the extent of an infection) and tolerance (the ability to tolerate the physiological consequences of an infection) are the two large categories of host defense mechanisms that affect evolutionary outcomes for symbioses (Simms and Triplett 1994; Råberg et al. 2007; Ayres and Schneider 2012). The equivalent symbiont virulence mechanisms are proliferation (the ability to proliferate to a certain extent in an infection) and benevolence (the ability to cause beneficial or harmful physiological consequences of an infection) (Wollein Waldetoft et al. 2020). Given a specific combination of host and symbiont, variable host resistance and symbiont proliferation lead to variation in symbiont density, while variable host tolerance and symbiont benevolence lead to variation in host fitness consequences relative to symbiont density (Figure 1). The evolution of host resistance and tolerance have epidemiological consequences, as host resistance often limits the spread of infections while host tolerance can do the opposite and cause infections to

increase in frequency in a population (Roy and Kirchner 2000; Råberg 2014). The evolution of symbiont benevolence and proliferation have significance in therapeutic drug development, as targeting pathogen benevolence rather than proliferation has been suggested as a potential way to prevent counter adaptation such as the evolution of antimicrobial resistance (Wollein Waldetoft et al. 2020).

The amoeba *Dictyostelium discoideum* is an established model for understanding intracellular pathogen infections (Steinert and Heuner 2005). It forms a facultative symbiosis with three species of *Paraburkholderia*: *P. agricolaris*, *P. bonniea*, and *P. hayleyella* (Brock et al. 2020). Roughly one quarter of soil-isolated *D. discoideum* strains carry one or occasionally multiple species of these symbiotic *Paraburkholderia* (Haselkorn et al. 2019). All three species of *D. discoideum*-associated *Paraburkholderia* are unique among close relatives in their ability to persistently infect *D. discoideum* (DiSalvo et al. 2015; Haselkorn et al. 2019). But two of the *Paraburkholderia* symbionts have genomes that are half the size of the third (Brock et al. 2020; Noh et al. 2022). Longer term symbionts tend to have smaller genomes compared to free-living relatives (Mira et al. 2001; Weinert and Welch 2017). While *P. agricolaris* is difficult to distinguish from other *Paraburkholderia* species based on its genome size and content, comparative genomics analyses indicate that *P. bonniea* and *P. hayleyella* have evolved reduced genomes in the amoeba host environment (Noh et al. 2022).

The fitness outcomes of *D. discoideum-Paraburkholderia* association appear to be context-dependent as with most facultative host-microbe symbioses (Drew et al. 2021; Husnik et al. 2021). *D. discoideum* hosts generally suffer negative fitness consequences of association. Amoebas infected with *Paraburkholderia* symbionts in the lab tend to eat less food bacteria during vegetative growth, migrate shorter distances as slugs during their multicellular social stage, form shorter and smaller volume fruiting bodies, produce fewer spores, and carry other bacteria alive (secondary carriage) into their next vegetative growth stage (Brock et al. 2011; DiSalvo et al. 2015; Shu et al. 2018; Miller et al. 2020). Potential context-dependent fitness benefits to the host appear to be an improved competitive ability against other *D. discoideum* strains, hypothetically by passing on symbiont infections or releasing *Paraburkholderia* secretions in a defensive manner (Brock et al. 2013). We know less about fitness outcomes of association for *Paraburkholderia* symbionts but *P. hayleyella* (though not *P. agricolaris*) reaches higher densities in the presence of *D. discoideum* compared to on its own in soil medium (Garcia et al. 2019).

Our goal was to understand whether naturally occurring (native) host-symbiont strain pairs within one of the reduced genome species, *P. bonniea*, is the result of host-symbiont coevolution. We use the term native host to indicate a *D. discoideum* strain from which one of the three *Paraburkholderia* symbiont species were isolated or identified. If native host-symbiont combinations lead to higher fitness estimates for host and symbiont compared to nonnative combinations, we assumed a stronger chance that coevolution had led to their co-occurrence. Patterns of coevolution were previously tested for the two other *D. discoideum* symbionts, *P. agricolaris* and *P. hayleyella*, but not for *P. bonniea*. In response to infection with *P. hayleyella*, nonnative and native *P. agricolaris* hosts had a higher fitness cost compared to native *P. hayleyella* hosts. In contrast, nonnative hosts, native *P. agricolaris* hosts, and native *P. hayleyella* hosts respond with similarly decreased fitness to *P. agricolaris* infection (Shu et al. 2018). *P. hayleyella* had higher abundance in soil with *D. discoideum* than soil without, while *P.*

agricolaris abundance was not affected by *D. discoideum* presence (Garcia et al. 2019). Both studies suggest that coevolution has occurred for *P. hayleyella* hosts and symbionts but not for *P. agricolaris* hosts and symbionts. Because *P. hayleyella* has a reduced genome, as does *P. bonniea*, we expected to see support for coevolution in our study.

D. discoideum has single cell and multi-cell stages in its life cycle. Vegetative single cell amoebas feed on bacteria until food density becomes low and starvation begins. Now in their social stage, starving amoebas secrete a chemical signal that causes amoebas within range to aggregate toward higher concentrations of this signal. This leads to aggregation, which is followed by transformation of the aggregated group into a multicellular body that ultimately forms fruiting bodies. The amoebas that survive the social stage become spores contained within the fruiting body sorus. In the lab, both stages of the life cycle occur as *D. discoideum* spores are allowed to hatch on petri dishes with food bacteria. Vegetatively multiplying amoebas will eventually exhaust their available food, aggregate, and ultimately form fruiting bodies after going through a social stage *in situ*.

We used both vegetative and social stages of this life cycle to approximate interactions between *D. discoideum* hosts and *P. bonniea* symbionts in an environment in which hosts form social groups and symbiont horizontal transmission is facilitated by these social groups. We used the vegetative stage to expose *D. discoideum* amoebas to *P. bonniea* symbionts, and used spore counts after an unmanipulated social stage as a measure of host fitness. We then used a subsequent social stage to expose uninfected amoebas to pre-infected amoebas, and estimated symbiont horizontal transmission after this manipulated social stage as an important aspect of symbiont fitness. We estimate host fitness and symbiont transmission at the social group level rather than at the individual amoeba host level using the social stage of the *D. discoideum* life cycle. Our estimates of symbiont density (e.g. Figure 1) is therefore better described as symbiont infection prevalence within a group of amoebas, and host fitness is also not a direct measure of individual host mortality but instead a relative measure of group productivity given a degree of symbiont prevalence. The same is true for symbiont transmission and is an estimate of transmission that occurs within a social group of interacting hosts rather than transmission from one host to another, as our estimates are a measure of newly added symbiont infection prevalence in a group of amoebas given a previous degree of symbiont infection prevalence.

Methods

Focal host and symbiont strains

We used three native host strains of *P. bonniea* (QS395, QS433, QS859) and three nonnative *D. discoideum* strains (QS4, QS17, QS18). All *D. discoideum* strains were previously isolated from Mountain Lake Biological Station in Virginia, USA. The native host strains had been cured of their symbionts using tetracycline and verified as symbiont-free using PCR. We used three *P. bonniea* symbiont strains (BbQS395, BbQS433, BbQS859). For each replicate, host (with food bacteria *Klebsiella pneumoniae*) and symbiont strains were grown from freezer stock on SM/5 plates (2 g glucose, 2 g BactoPeptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g $MgCl_2$, 1.9 g KH_2PO_4, 1 g K_2HPO_4 and 15 g agar per liter). KK2 buffer (2.2 g KH2PO4 monobasic and 0.7 g K2HPO4 dibasic per liter) was used throughout for handling bacteria, *D. discoideum* spores and amoebas.

Host fitness

To estimate host fitness at a range of infection prevalence, *D. discoideum* spores from freshly grown fruiting bodies were collected and placed on SM/5 plates at a density of 2 x 10^5 spores per plate and RFP-labeled symbionts at multiplicities of infection (MOI) of 0 (control), 0.6, 3, and 15. We made three replicate plates per combination and left these plates to fruit at 21 C for 5-7 days. We then collected all fruiting bodies from each plate and estimated the number of spores by counting diluted spores on a hemocytometer and multiplying these counts by the total volume of spores. We then took the percent of spores from each host-symbiont combination relative to the uninfected control that was prepared at the same time. For example, host strain QS4 was infected with BbQS395 at MOI of 0 (control), 0.6, 3, and 15. Spore counts of each individual sample plate were divided by the average spore count of the three uninfected control plates. Because our models examine the relationship between host fitness and infection prevalence (not MOI), we estimated infection prevalence (percent of RPF+ infected spores in a sample of 100,000) by running a sample of spores on a BD FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ) to.

Symbiont transmission

To estimate symbiont horizontal transmission during a social stage, *D. discoideum* spores from freshly grown fruiting bodies were collected and placed on SM/5 plates at a density of 2 x 10^5 spores per plate with RFP-labeled symbionts at MOI of 0 (control), plus an additional 3-5 MOI ranging from 0.3 to 30. Once these plates had fruited and amoebas were pre-infected, we collected fresh spores and placed them on SM/5 plates at densities of 1 x 10^5 and 2 x 10^5 spores per plate with only food bacteria. When amoebas were at log-phase growth roughly 36 hours later, uninfected amoebas were dyed with CellTracker Green CMFDA (Invitrogen) dissolved in DMSO. Pre-infected amoebas (RFP+) were carried along with the dyed amoebas, only exposed to DMSO, and treated in the same way through room temperature incubation and washes. Afterward, dyed uninfected amoebas were combined with infected amoebas at 1:0 (dyed only negative control), 0:1 (infected only), and 1:1 (mixed) ratios and placed on moistened nitrocellulose filters to aggregate and develop into fruiting bodies in replicates of three. 5-7 days later, we collected all fruiting bodies from these filters and ran these samples on a BD FACSCalibur flow cytometer. We estimated infection prevalence from the infected only sample and horizontal transmission from the mixed sample as indicated by the co-occurrence of dye and infection in a spore. FCS files were imported into FlowJo v10.8.1 for analysis. We applied gates for spores, then dyed cells from the negative control, and infected cells from the highest MOI sample. A logical (AND) gate was applied to the 1:1 mixed sample to find the percent of spores that were previously uninfected amoebas but were now positive for symbiont infection (Figure 2).

Statistical analysis

Statistical analyses were performed in R v3.6.0 and with packages car v.3.0-12 (Fox and Weisberg 2019) and lme4 v.1.1-28 (Bates et al. 2015). For both host fitness and symbiont transmission, we fit linear models with mixed effects. For host fitness, we tested how percent spores (relative percent of spores produced) were affected by symbiont strain and host type

(native or nonnative). We tested the effect of host type by coding the native combinations either strictly (e.g. only QS859 infected with BbQS859 would be considered native) or leniently (e.g. any native host of *P. bonniea* infected with any *P. bonniea* strain is considered native). We used infection prevalence as a continuous predictor and experiment date and host strain as random effects. For symbiont transmission, we fit a similar model but with percent transmission (percent newly infected spores that were previously uninfected amoebas) as the dependent variable. We fit the most complex model first, then removed nonsignificant terms and compared the simpler model with the complex one using a chi-squared test. We accepted the simpler model if no significant difference was found in model fit. We examined residuals of the final model for any indications that we had violated model assumptions. We examined effect sizes of factors in the final model using the package effectsize v.0.7.0 (Ben-Shachar et al. 2020) and its epsilon_squared() function. We also compared infection prevalence across the two experiments. We fit linear models with host strain as a random effect and the different number of social stages (nonnumeric factor; one for host fitness, two for symbiont transmission) and symbiont identity as fixed effects. We examined residuals of the final model for any indications that we had violated model assumptions and examined effect sizes of factors.

Data availability

Data and R code supporting this work can be accessed at: https://github.com/noh-lab/social-infection

Results

Host fitness

We estimated the relationship between host fitness and symbiont infection prevalence by exposing native and nonnative *D. discoideum* hosts to three strains of *P. bonniea* at a range of multiplicities of infection. Host fitness decreased with increasing infection prevalence across all combinations of hosts and symbionts that we tested, but there was no difference between native and nonnative hosts (Figure 3; Table 1). This was regardless of whether native host status was defined using a strict or lenient definition (Table S1). Instead, symbiont strain identity significantly affected host fitness. For example, BbQS859 infections resulted in a shallower slope in host fitness decline compared to BbQS433 infections which resulted in a steeper slope (Figure 3). These results support a significant effect of symbiont benevolence variation on host fitness during initial infection. Based on our results, BbQS859 is more benevolent while BbQS433 is more malevolent to any type of host. BbQS395 appears to show an intermediate benevolence phenotype.

Symbiont transmission

We estimated the relationship between symbiont transmission and initial symbiont infection prevalence by exposing uninfected amoebas to pre-infected amoebas during a manipulated social stage of the host life cycle and detecting the percentage of newly infected spores. The degree of symbiont horizontal transmission increased with the infection prevalence of pre-infected amoebas that entered each social stage (Figure 4). This positive correlation confirms that the increase in infection is due to horizontal transmission rather than vertical

transmission (Ebert 2013), which is what we expected because very few amoebas go through cell division during the social stage (Muramoto and Chubb 2008). As with host fitness above, native and nonnative hosts were no different in overall patterns of symbiont transmission, regardless of how native hosts are defined (Table 2). However, the degree and slope of symbiont transmission was affected by symbiont strain identity, indicating that both symbiont proliferation and benevolence affect symbiont transmission (Figure 4). For example, over more than a single social stage, BbQS859 has higher proliferation compared to BbQS395 and BbQS433. In addition, BbQS859 symbionts were transmitted at significantly higher levels compared to the other symbiont strains, and at a steeper slope beyond that simply due to BbQS859 infections occurring at higher infection prevalence. We also detected some differences in how infection prevalence, and host type interacted when we leniently defined native hosts. Howeve, much of this difference may be attributed to BbQS859, where nonnative hosts became infected at a slightly higher density compared to native hosts and therefore also transmitted symbionts at a higher level.

Synthesis

We observed a significant difference between infection prevalence in these two experiments. This is likely due to differences in symbiont strain-specific virulence, both in terms of proliferation and benevolence (Table 3; Figure 5). The main difference between the design of the two experiments is that for *Host fitness* we used newly-infected amoebas, but for *Symbiont transmission* we used pre-infected amoebas that had been infected in a previous vegetative stage so as to minimize the potential for symbionts to be newly acquired from the environment and to maximize our ability to detect horizontal transmission among amoebas. We infected amoebas for the symbiont transmission experiment at a wider range of MOI (0.3-30) compared to the host fitness experiment (0.6-15) in order to achieve as wide a range of infection prevalence as possible. We found that we were unable to push the maximum of these ranges higher by using a higher MOI for the initial infection. In fact, typically one of the intermediate MOI resulted in the highest observed infection prevalence after the amoebas had gone through a social stage.

Our combined observations from both experiments suggest that variation in symbiont benevolence leads to variation in symbiont proliferation among *P. bonniea* strains infecting amoeba hosts. More malevolent strains (BbQS395 and BbQS433) proliferate less among amoeba hosts compared to more benevolent strains (BbQS859) over the course of two vegetative stage - social stage cycles (Figure 5). This leads to less transmission of malevolent strains compared to benevolent strains during the next social stage (Figure 4).

Discussion

Host and microbe partners engaged in a facultative symbiosis are not mutually dependent on each other for survival as are partners in an obligate symbiosis. Yet stable associations between hosts and facultative symbionts clearly exist (Mondo et al. 2012; Werner et al. 2015). *P. bonniea*, along with *P. hayleyella* and *P. agricolaris*, is able to persistently infect *D. discoideum* and form a facultative symbiosis (DiSalvo et al. 2015; Haselkorn et al. 2019; Miller et al. 2020). Both *P. bonniea* and *P. hayleyella* have reduced genomes that are roughly half the size of *P. agricolaris*, leading to the hypothesis that both reduced-genome species may

have associated with *D. discoideum* for a longer period of evolutionary time compared to *P. agricolaris* (Brock et al. 2020; Noh et al. 2022), and thus forming a stable facultative symbiosis. Whereas we had expected that the presence of reduced-genome symbionts may likely be due to host-symbiont coevolution, our results indicate this may not be the case for hosts and *P. bonniea* symbionts. Our results from *P. bonniea* show the presence of variation in symbiont virulence mechanisms and a lack of variation in *D. discoideum* host defense mechanisms in response to *P. bonniea* infection. The fitness outcomes of infected hosts were no different between native and nonnative hosts. Overall, when more individual hosts were infected, the collective number of spores produced decreased for both types of hosts, indicating that infection with *P. bonniea* is a detriment to amoeba host fitness. We did find evidence for strain level variation in symbiont virulence mechanisms that affect symbiont transmission, by enabling increased horizontal transmission in benevolent strains compared to malevolent strains. Overall, when more individual hosts were infected symbionts were better able to infect additional hosts via horizontal transmission, suggesting that *P. bonniea* benefits from infecting more amoeba hosts.

Lack of variation in host defense mechanisms – ecology and host range of Paraburkholderia symbionts

Our results indicate that native hosts of *P. bonniea* have not evolved improved defense mechanisms in response to association with *P. bonniea* compared to nonnative hosts. We find this to be a curious result. Both *P. hayleyella* and *P. bonniea* have reduced genomes and are each other's closest relatives (Brock et al. 2020). Some evidence of host-symbiont coevolution has been observed for *P. hayleyella* and its native hosts, though with different experimental approaches (Shu et al. 2018; Garcia et al. 2019). *P. bonniea* had remained untested until now. Our post-hoc hypothesis for the lack of defense mechanisms in hosts of *P. bonniea* is related to the ecology of both host and symbiont. *P. bonniea* is the rarest of *Paraburkholderia* symbionts of *D. discoideum* (Haselkorn et al. 2019; DuBose et al. 2022) and it also appears to be the symbiont species that is least detrimental to host fitness on average (Miller et al. 2020). Rare encounters between new hosts and *P. bonniea* may not have a significant impact on *D. discoideum* populations, particularly if *D. discoideum* itself is sparsely distributed across soil landscapes. If both host and symbiont are rare, we expect the fitness cost of infection for the relatively benign *P. bonniea* to be insufficient for hosts to evolve defense mechanisms against it (Anderson and May 1982).

The apparent lack of evolved host defense mechanisms in native hosts of *P. bonniea* brings us to an interesting question regarding *Paraburkholderia* symbionts of *D. discoideum* social amoebas: are they generalist or specialist symbionts to *D. discoideum*? *P. agricolaris* and other *Burkholderia* sensu lato appear to frequently infect Dictyostelid and Acytostelid social amoeba species (Sallinger et al. 2021). Comparison between soil microbiomes and amoeba microbiomes found that *Burkholderia* are present in soil samples but are orders of magnitude more abundant in amoebas. This increased abundance suggests that bacteria that can associate with social amoebas are benefiting from being separated from various environmental stresses and competition from other soil bacteria, and potentially also adapted to living inside social amoebas. These pieces of evidence indicate that *P. agricolaris* may be a generalist symbiont of social amoebas, and *P. agricolaris* infection from the environment and horizontal

infection between amoebas may be common. The rarer *P. bonniea* and *P. hayleyella* are still elusive in terms of their natural host ranges (DuBose et al. 2022). But given their relative scarcity and difference in genome size, we predict that both reduced-genome species are more likely to be specialists of *D. discoideum*. The host range of a symbiont is affected by ecological factors including host density and relatedness, symbiont transmission dynamics, and the presence of additional symbionts competing for access to host resources (Barrett et al. 2009; Leggett et al. 2013). The projected ecological differences between *P. agricolaris* and the reduced genome species *P. bonniea* and *P. hayleyella* appear to be consistent with previous observations that generalist symbionts (potentially *P. agricolaris*) are more likely to have environmental origins while specialists (potentially *P. bonniea* and *P. hayleyella*) are more likely to have been commensals evolving within a host species (Brown et al. 2012; Bäumler and Fang 2013).

Strain level variation in symbiont virulence mechanisms – virulence-transmission trade-off

We find evidence that strains of *P. bonniea* vary in both symbiont virulence mechanisms of proliferation and benevolence. We consider the virulence-transmission trade-off to contextualize this observed variation in the interaction between *D. discoideum* and *P. bonniea*. Despite the commonly-held expectation that relatively benign symbionts will result from long-term host-symbiont coevolution, the virulence-transmission trade-off hypothesis does not have a guaranteed expectation of what will happen to symbiont virulence per se. Instead, the trade-off hypothesis posits that if there is a biological link between symbiont virulence and transmission (because symbionts use host resources to replicate), symbionts should evolve toward a level of virulence where transmission is greatest (Anderson and May 1982). This hypothesized trade-off has proven difficult to observe for several reasons, including the influences of host range, multiple symbiont infections, and host population structure that can affect host-symbiont interactions in complex ways (Alizon et al. 2009; Leggett et al. 2013). However, recent meta-analyses indicate that there is partial support for the trade-off hypothesis in several biological systems (Acevedo et al. 2019). The three core predictions of the trade-off hypothesis are as follows: (i) within-host symbiont replication rates have a positive relationship with symbiont virulence; (ii) within-host symbiont replication rates have a positive relationship with symbiont transmission rates; and (iii) symbiont virulence and transmission have a trade-off through the negative relationship between symbiont virulence and host recovery rates. Diverse study systems show support for (i) and (ii), but only a nonsignificant trend for (iii) (Acevedo et al. 2019).

Because we estimated host fitness and symbiont transmission at the social group level rather than at the individual amoeba host level, our evidence that supports each of these points may be considered an indirect test of the virulence-transmission trade-off as the original theory is built on investigating symbiont density in individual hosts. That said, we find support for each of the core predictions of the virulence-transmission trade-off hypothesis. Symbiont infection prevalence had a negative relationship with host fitness (i; Figure 3), and symbiont infection prevalence had a positive relationship with symbiont horizontal transmission (ii; Figure 4). Lastly, variation in symbiont benevolence led to increased horizontal transmission of benevolent strains compared to malevolent strains (iii; Figure 4). The patterns of symbiont virulence mechanisms we observed also predict that more benevolent *P. bonniea* strains should be found

at higher frequencies given similar conditions compared to more malevolent strains. Because of the relative scarcity of *P. bonniea* in nature, we may never observe the ecological consequence of this trade-off we have detected in the lab. However the presence and extent of horizontal transmission in *P. bonniea* indicates that this symbiont benefits from infecting *D. discoideum* hosts by both proliferating within a given host and transmitting to new hosts.

We expect transmission dynamics to be an important factor in further understanding *P. bonniea* and *P. hayleyella* evolution because of the complex interaction between host specificity, symbiont virulence, and symbiont transmission. Given the persistence of *Paraburkholderia* symbiont infection in the lab and the facultative aspect of the symbiosis itself, we expect these symbionts to transmit among hosts using both vertical and horizontal routes. While we did not test for vertical transmission, our results clearly show the presence of significant horizontal symbiont transmission among *D. discoideum* amoebas in the social stage of their life cycle. The coexistence and relative influences of both types of transmission routes are better understood in other amoeba-bacteria symbioses. For example, *Acanthamoeba* hosts interact with *Parachlamydia acanthamoebae* symbionts across the cyclical life cycle of the symbiont (Herrera et al. 2020). The symbiont can replicate within a host-derived vacuole in the form of reticulate bodies that are also vertically transmitted from parent to daughter cells. *Parachlamydia* symbionts are released into the environment in the form of elementary bodies that do not replicate but can horizontally infect new hosts.

Coevolution or random co-occurrence of host and symbiont in a facultative symbiosis

Our results from *P. bonniea* show the presence of variation in symbiont virulence mechanisms and a lack of variation in *D. discoideum* host defense mechanisms between native and nonnative hosts. Therefore, naturally occurring host-*P. bonniea* strain pairs do not appear to be the result of evolved host defense mechanisms in native hosts compared to nonnative hosts. The presence of phenotypic variation among symbiont strains and in some host-symbiont combinations indicates that adaptation is potentially ongoing for *P. bonniea* symbionts. In contrast the lack of variation among *D. discoideum* host types can indicate a lack of adaptation potential but can also be the result of host defense mechanism fixation that has occurred post-adaptation. A lack of adaptation potential in host defense mechanisms against *P. bonniea* may be because *P. bonniea* presents only a weak selection pressure, due to the mild reduction in fitness experienced by amoeba hosts even at high infection prevalence and what we expect is the relatively infrequent encounter rate of *P. bonniea* by environmental *D. discoideum*. We plan to apply the experimental approach of the current study to the relationship between *D. discoideum* and its other reduced-genome symbiont species *P. hayleyella*, which is known to cause much more detrimental fitness consequences to newly infected hosts (Shu et al. 2018; Miller et al. 2020). Additional results from *P. hayleyella* should provide more insight into the patterns we observed using *P. bonniea*.

Attempts to find individual strain-level coevolution in facultative symbionts are rare. Niepoth and colleagues (2018) found that one particular *Hamiltonella defensa* symbiont strain caused detrimental fitness effects to nonnative pea aphid hosts compared to the native host. However, the detrimental fitness effects to nonnative hosts appeared to be transient, and did not continue after infection establishment. In addition, hosts from a population (*Lotus pedunculatus* biotype) that typically carry *H. defensa* were more likely to establish symbiosis when newly

infected compared to hosts from another population (*Lotus corniculatus* biotype). These results suggest that a potential reason why we did not find evidence of host adaptation is because our native and nonnative hosts were collected from the same geographical locality. Therefore, it is possible that *D. discoideum* hosts from a locality where *P. bonniea* symbionts are found have already adapted to their infection and host defense mechanisms have become fixed. At this time, it is unclear how to test whether the patterns we observed with *P. bonniea* are the result of post-adaptation fixation because we do not have a way to determine whether an amoeba collected from the field has encountered a *Paraburkholderia* previously.

Conclusions

The *D. discoideum*-*Paraburkholderia* system continues to provide opportunities for investigating the evolution of a naturally occurring facultative symbiosis between a protist host and bacterial symbionts. In addition, we find this system to be particularly valuable in that it presents an opportunity to study symbiosis in the context of social groups of hosts, so that we can readily assess the extent and impact of symbiont horizontal transmission on both host and symbiont. In summary, our work shows that *P. bonniea* possesses variable phenotypes in symbiont proliferation and benevolence, whereas native and nonnative hosts of *P. bonniea* do not differ in host defense mechanisms in response to association with *P. bonniea*. It is difficult to extrapolate what kind of evolutionary consequences may arise based on differences in experimentally estimated fitness effects in microbes, because variation in microbial population size can easily range across several orders of magnitude (Sheppard et al. 2018). However, the presence and extent of horizontal transmission in *P. bonniea* suggests that this symbiont benefits from infecting *D. discoideum* hosts by both proliferating within a given host and transmitting to new hosts. More importantly, we find support for the virulence-transmission trade-off, as variation in symbiont benevolence led to increased horizontal transmission of benevolent strains compared to malevolent strains during the social stage of the host life cycle. In contrast, native *D. discoideum* hosts do not appear to have a fitness advantage over nonnative *D. discoideum* hosts in their interactions with *P. bonniea*. Therefore, naturally occurring host-*P. bonniea* strain pairs do not appear to be the result of coevolution between host and symbiont.

Acknowledgements

We thank past and current members of the Noh lab (Caroline Lunt, Gus Shuster) who contributed moral support for this project during its development. Ron Peck, Laura Runyen-Janecky, and Chris Moore provided helpful comments that improved previous drafts.

This work was supported by the National Institutes of Health and its National Institute of General Medical Sciences by an Institutional Development Award (IDeA) under Grant Number P20GM103423 (subaward to SN) and Colby College startup funds.

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Tables and Figures

Table 1. Analysis of Deviance table for Host fitness with lenient native definition

Variable	X^2	***df***	***P-value***	η^2_p	***95% CI***
Infection prevalence	376.002	1	< 0.001	0.63	(0.56, 1)
Symbiont strain	17.998	2	< 0.001	- 0.06	(0, 1)
Host type	< 0.001	1	0.981	- 0.37	(0, 1)
Infection prevalence : Symbiont strain	23.886	2	< 0.001	0.09	(0.04, 1)

Table 2. Analysis of Deviance table for Symbiont transmission with lenient native definition

Variable	X^2	***df***	***P-value***	η^2_p	***95% CI***
Infection prevalence	484.965	1	< 0.001	0.77	(0.71, 1)
Symbiont strain	149.430	2	< 0.001	0.43	(0.20, 1)
Host type	2.092	1	0.148	0.53	(0.25, 1)
Infection prevalence : Symbiont strain	109.289	2	< 0.001	0.65	(0.52, 1)
Infection prevalence : Host type	35.991	1	< 0.001	0.30	(0.17, 1)
Symbiont strain : Host type	9.509	2	0.009	0.14	(0.01, 1)

Table 3. Analysis of Deviance table for infection prevalence across experiments

Variable	X^2	***df***	***P-value***	η^2_p	***95% CI***
Number of social stages	71.196	1	< 0.001	0.16	(0.11, 1)
Symbiont strain	17.789	2	< 0.001	0.02	(0.00, 1)
Number of social stages : Symbiont strain	39.197	2	< 0.001	0.09	(0.05, 1)

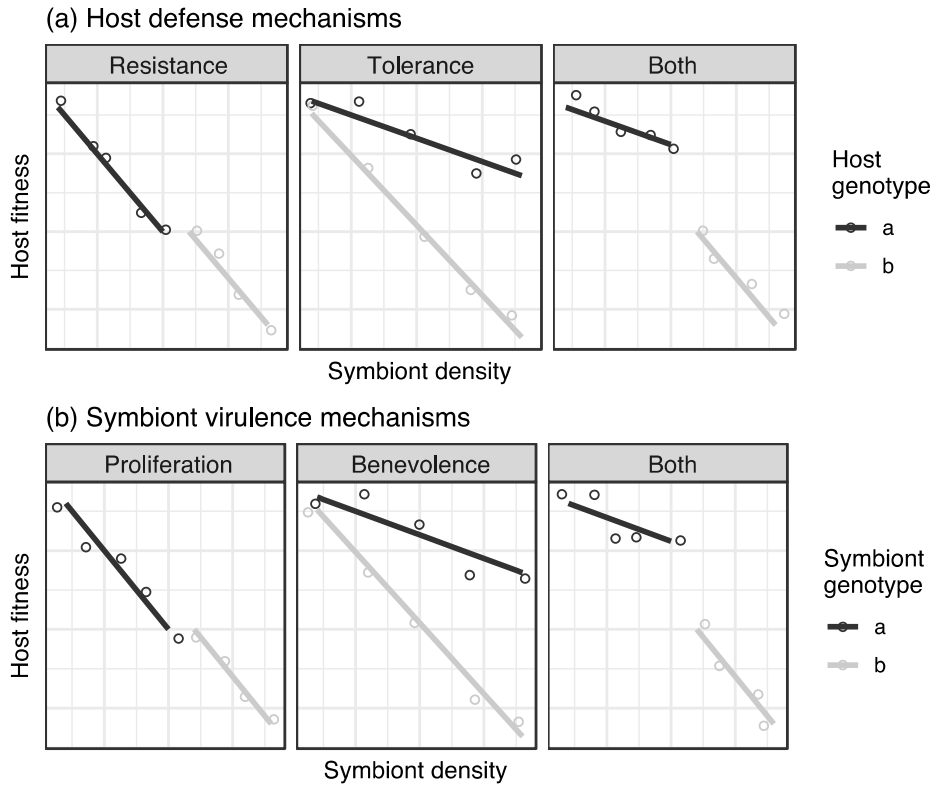


Figure 1. Theoretical differences between resistance-based and tolerance-based host defense mechanisms, and proliferation-based and benevolence-based symbiont virulence mechanisms. Both types of variation can be detected by measuring host fitness and detecting when (a) different host genotypes vary in responses to infection by the same symbiont, or (b) the same host genotype varies in responses to infection by different symbiont genotypes. (a) Hosts may vary in **resistance** to the same symbiont. More resistant hosts (a – black line; lower average symbiont density) will have overall higher fitness compared to less resistant hosts (b – gray line; higher average symbiont density). But the fitness cost per increase in symbiont density is similar for both hosts, as indicated by the similar slopes. On the other hand, hosts may vary in **tolerance** to the same symbiont. More tolerant hosts (a – black line; shallower slope) may have a similar average symbiont density to less tolerant hosts (b – gray line; steeper slope), but will suffer a lower fitness cost per increase in symbiont density. (b) The same host may suffer different fitness effects due to symbionts that vary in **proliferation**. The better proliferating symbiont (b – gray line; higher average symbiont density) will impart higher fitness costs to the host than the worse proliferating symbiont (a – black line; lower average symbiont density). But the fitness cost to the host per increase in symbiont density is similar for both symbionts. The same host may be infected with symbionts that vary in **benevolence**. More benevolent symbionts (a – black line; shallower slope) will impart a lower fitness cost per increase in symbiont density compared to more malevolent symbionts (b – gray line; steeper slope), though both symbionts may reach similar average densities. (Framework adapted from Raberg et al 2007 and Wollein Waldetoft *et al.*, 2020.)

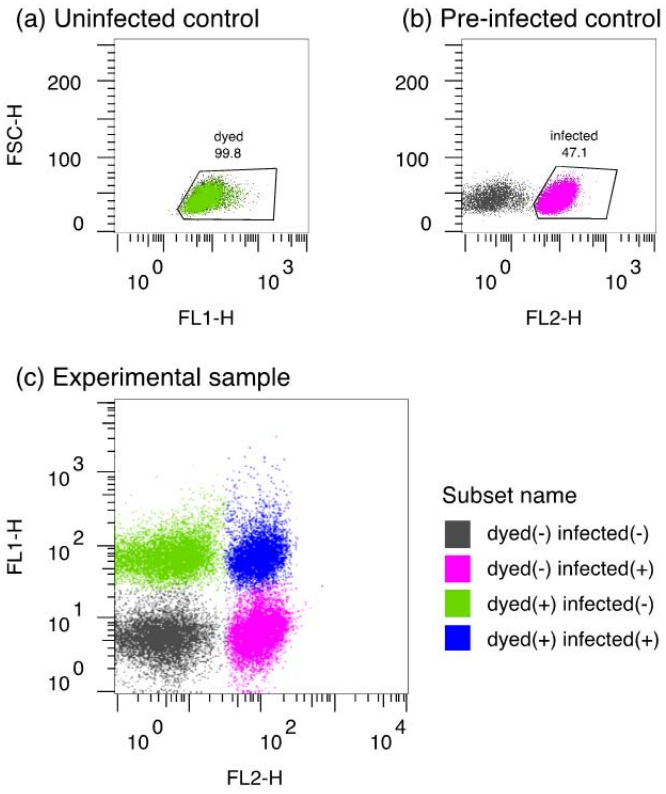


Figure 2. Representative samples from symbiont transmission experiment to demonstrate how symbiont transmission during a social stage is detected. (a) Dyed cells (green population) are gated on uninfected controls treated with Celltracker. (b) Infected cells with RFP-labeled symbionts (pink population) are gated on pre-infected controls. (c) Newly infected cells (blue population) are detected by applying logical gates to the experimental samples that mixed uninfected cells and pre-infected cells.

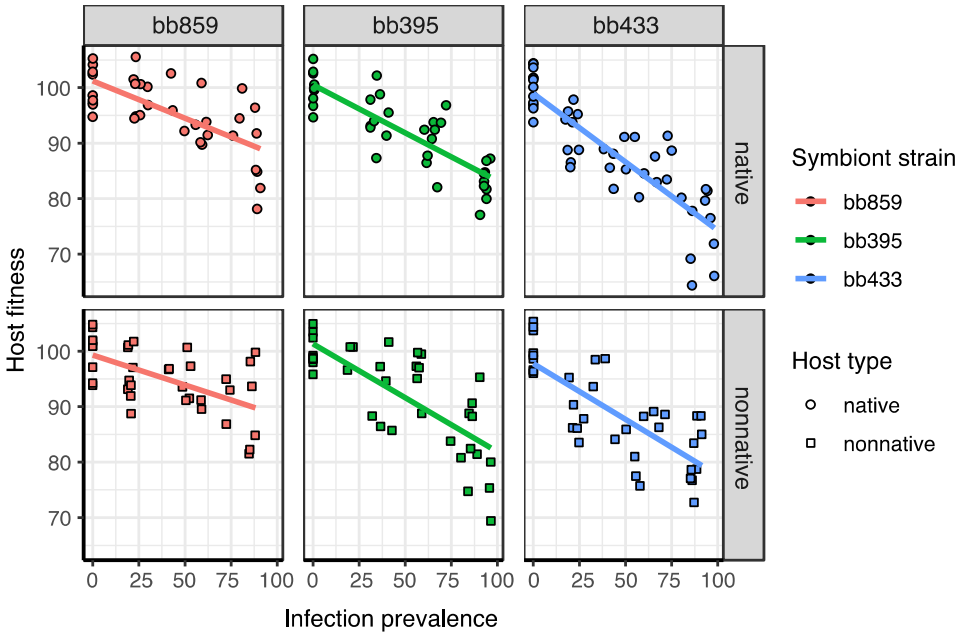


Figure 3. Host fitness was negatively correlated with infection prevalence and differed in slope but not average infection prevalence among symbiont strains after a single social stage. Infection prevalence was estimated per host-symbiont-MOI sample as the percent of infected spores that had RFP labeled symbionts in them. Host fitness was estimated by the percent of spores produced per host-symbiont-MOI sample, relative to uninfected hosts prepared at the same time as the infected host-symbiont combinations. There was no significant difference between native (top row; lenient definition) vs. nonnative (bottom row) hosts.

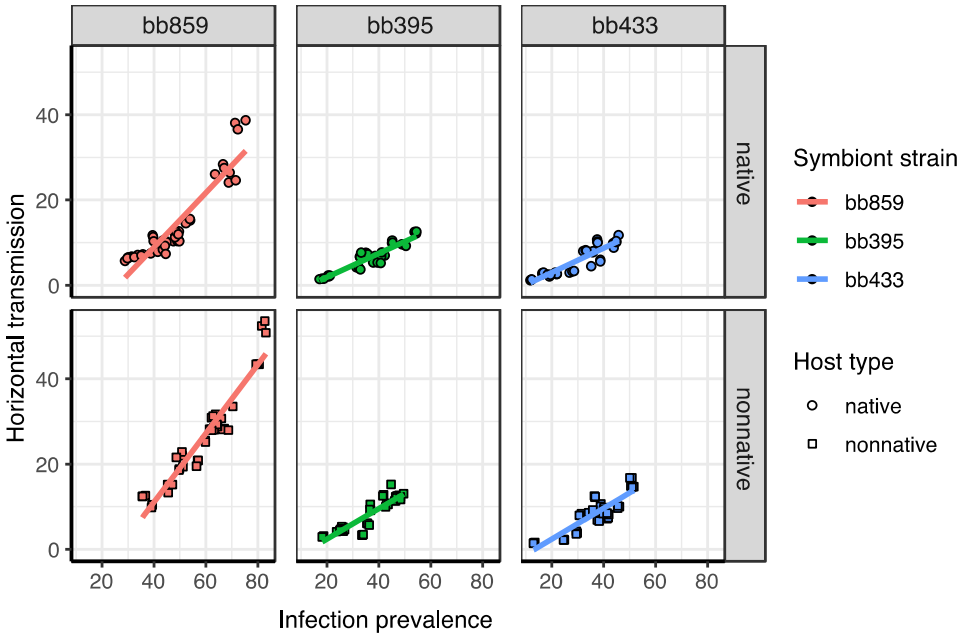


Figure 4. The rate of horizontal transmission was positively correlated with pre-infection prevalence and also differed in slope and average infection prevalence among symbiont strains after a second social stage. Infection prevalence was estimated per host-symbiont-MOI sample as the percent of RPF+ infected spores of the pre-infected control. Horizontal transmission was estimated by the percent of RFP+ infected spores in the test sample that were also positive for a green membrane dye. These dyed cells were uninfected prior to the experiment, during which they were combined with pre-infected (and undyed) cells. There was no significant difference in symbiont transmission among native (top row; lenient definition) and nonnative (bottom row) hosts.

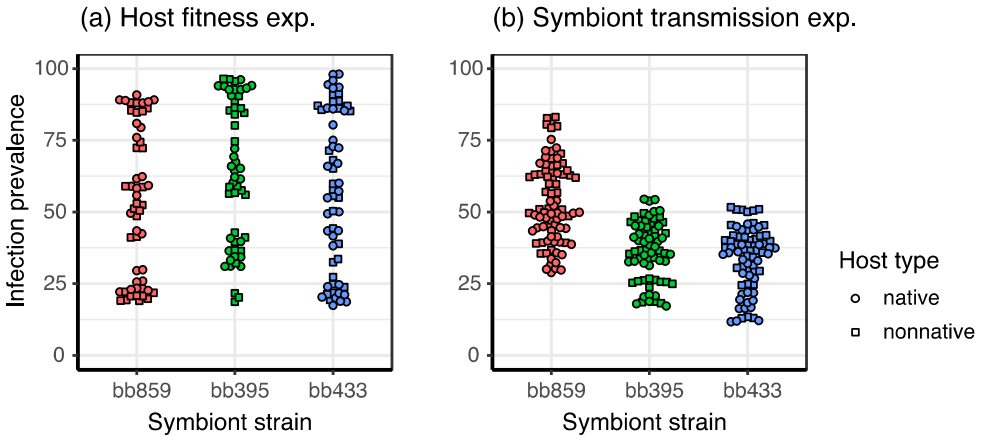
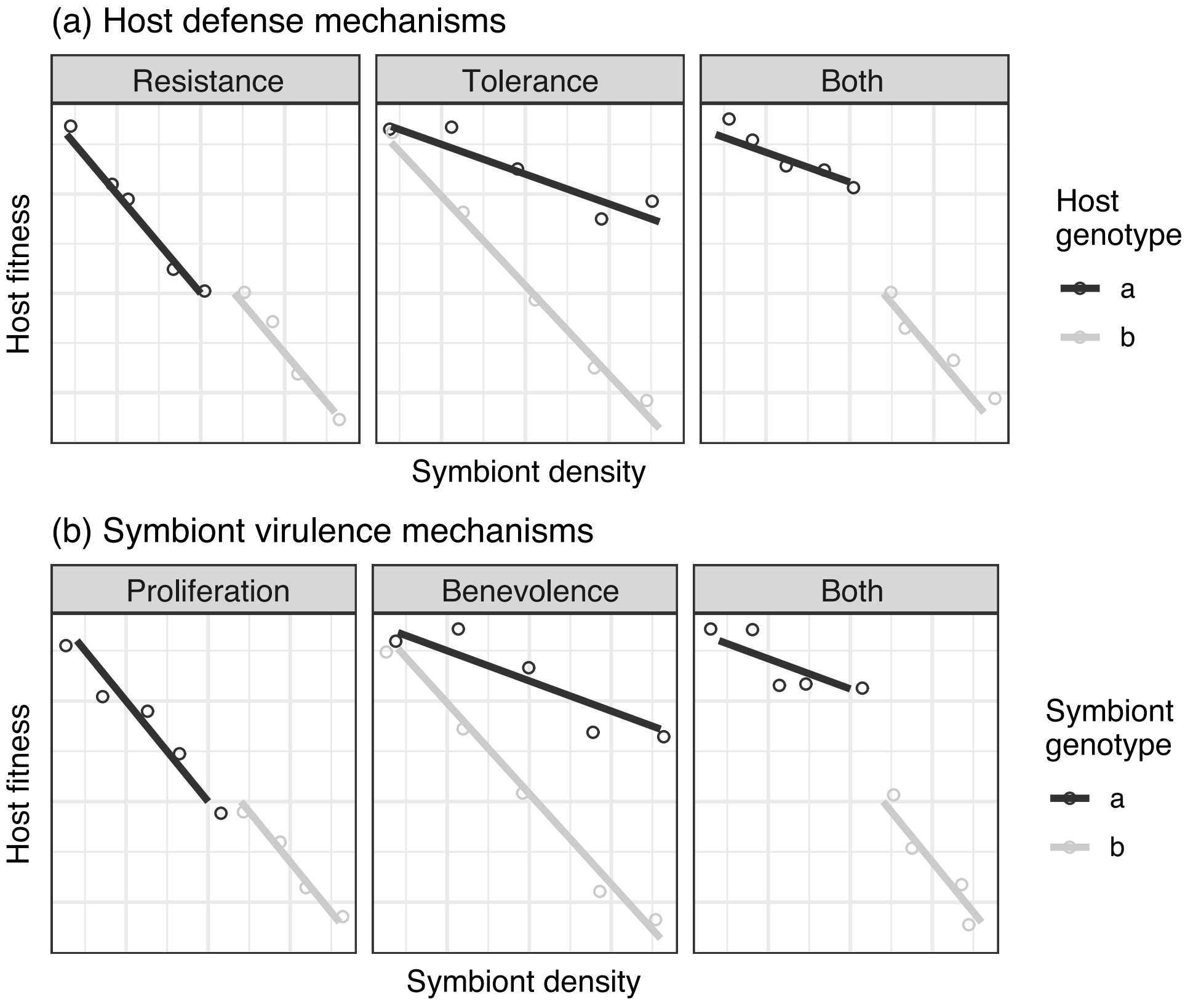
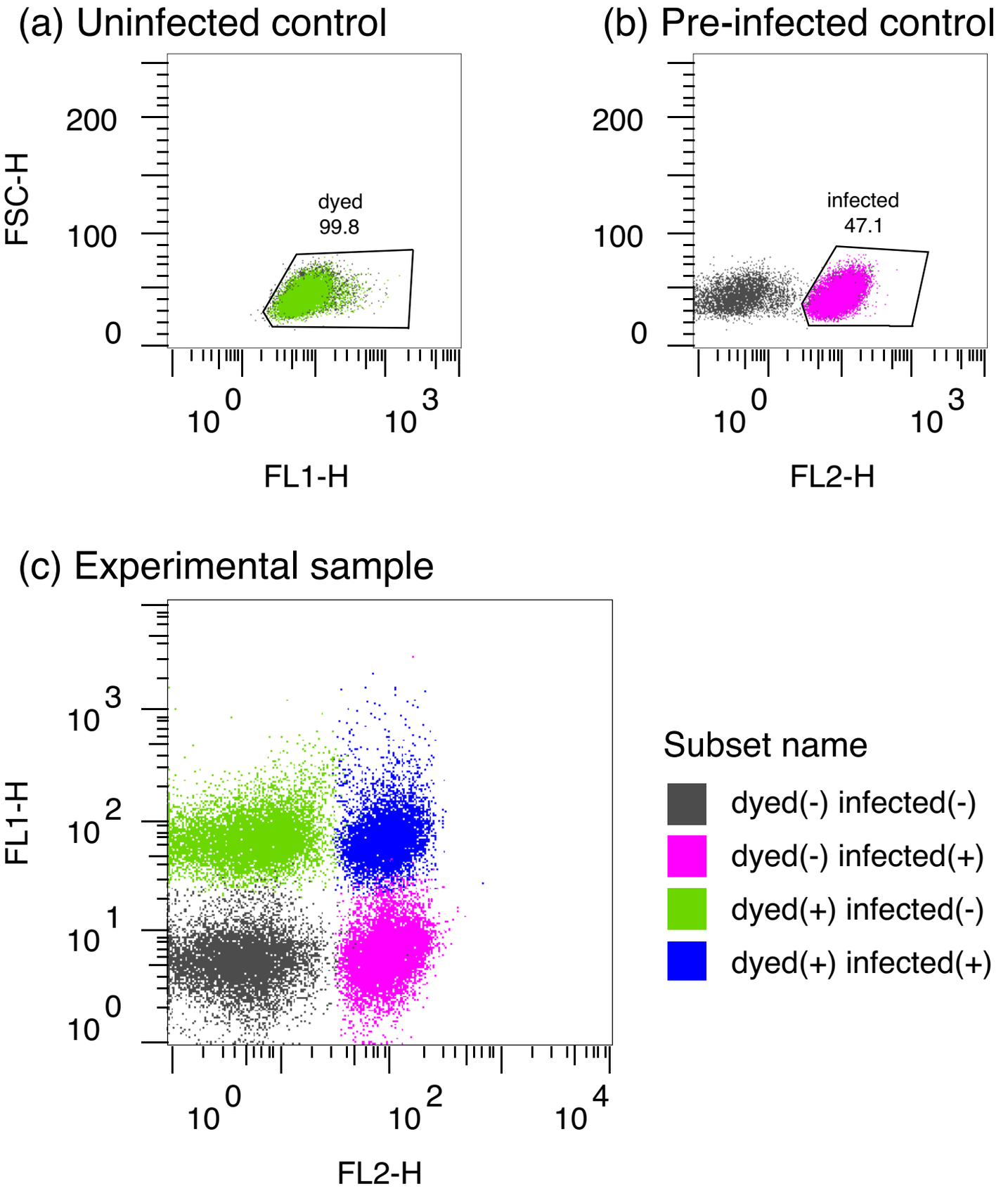
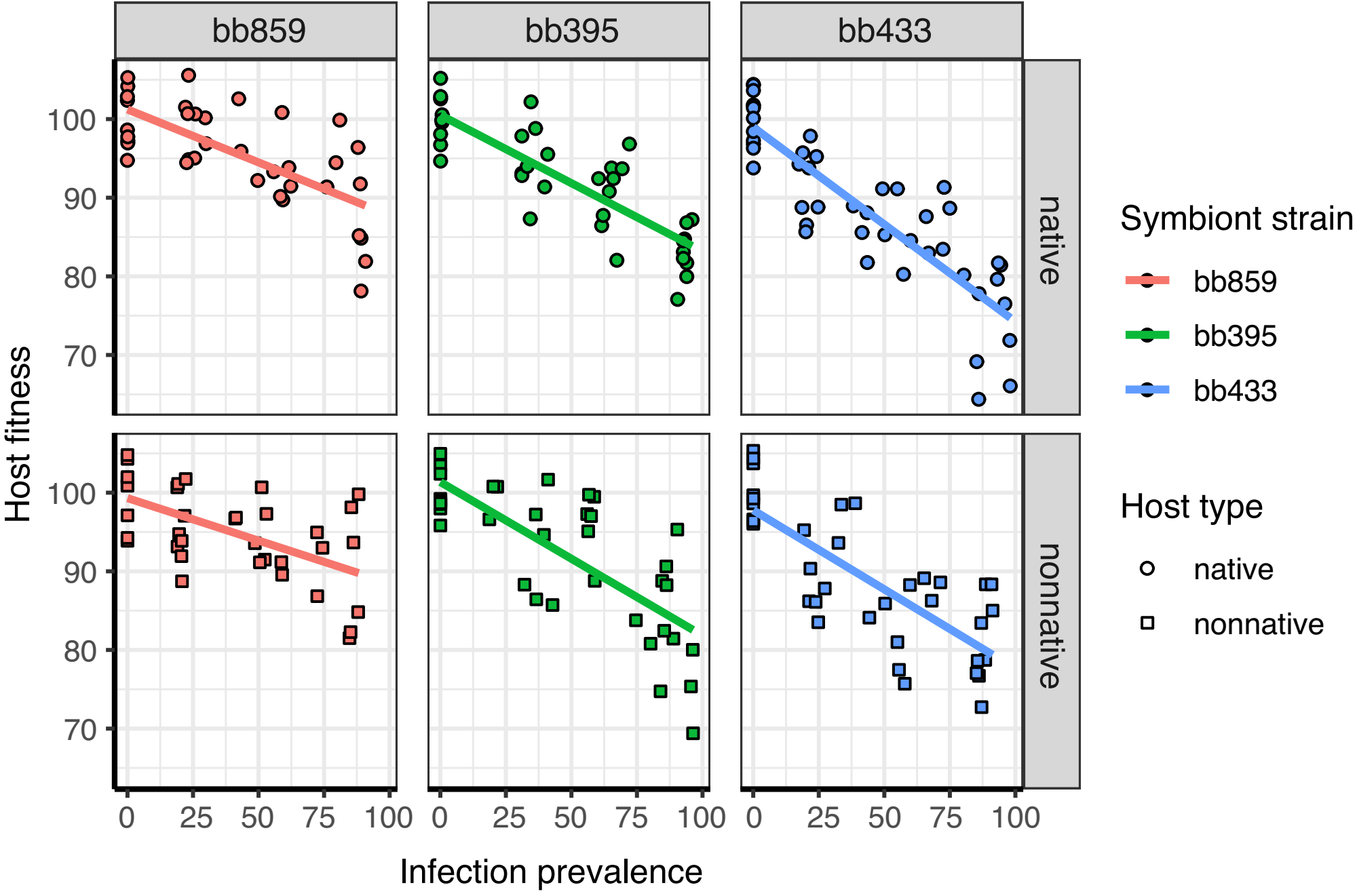
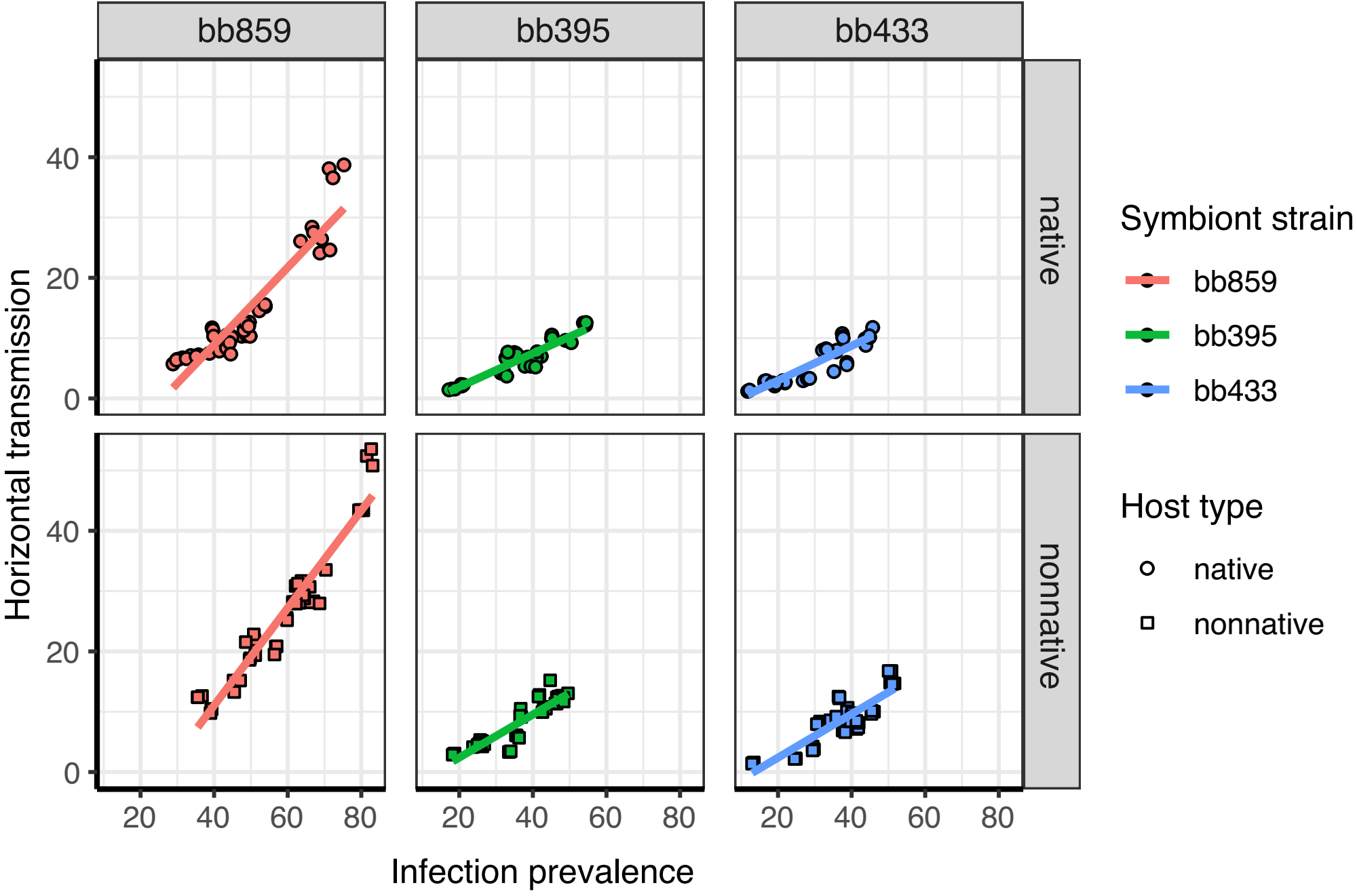


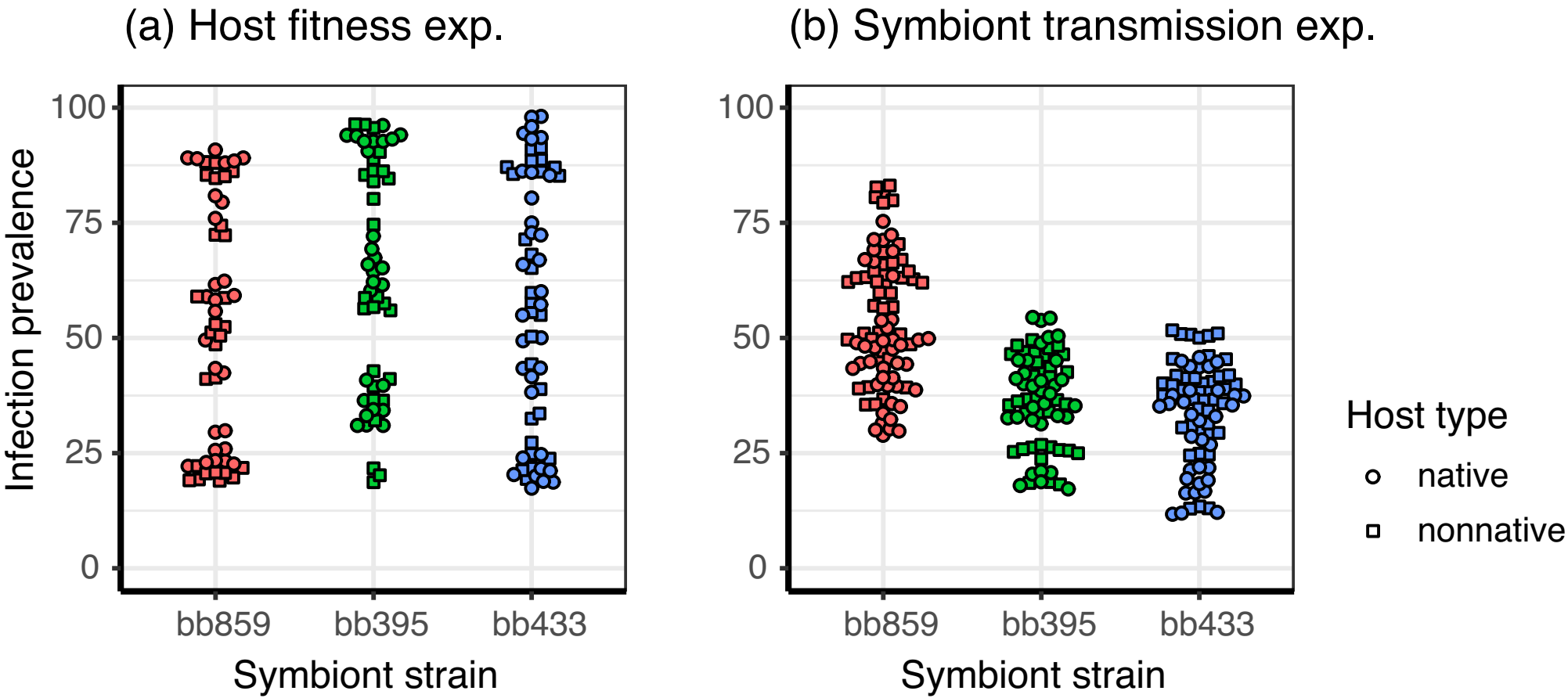
Figure 5. Infection prevalence was significantly different when amoebas were observed after one (a) vs. two social stages (b) after infection. More malevolent strains (BbQS395 and BbQS433) appear to proliferate less among amoeba hosts compared to more benevolent strains (BbQS859) over time.


(a) Host defense mechanisms
Resistance
Tolerance
Both
Host fitness
Symbiont density
Host genotype
a
b
(b) Symbiont virulence mechanisms
Proliferation
Benevolence
Both
Host fitness
Symbiont density
Symbiont genotype
a
b


(a) Uninfected control
FSC-H
200
100
0
dyed
99.8
10 0
10 3
FL1-H
(b) Pre-infected control
200
100
0
infected
47.1
10 0
10 3
FL2-H
(c) Experimental sample
FL1-H
10 3
10 2
10 1
10 0
10 0
10 2
10 4
FL2-H
Subset name
dyed(-) infected(-)
dyed(-) infected(+)
dyed(+) infected(-)
dyed(+) infected(+)


bb859
bb395
bb433
native
nonnative
Host fitness
100
90
80
70
0
25
50
75
100
Infection prevalence
Symbiont strain
bb859
bb395
bb433
Host type
native
nonnative


bb859
bb395
bb433
native
nonnative
Horizontal transmission
Infection prevalence
0
20
40
20
40
60
80
Symbiont strain
bb859
bb395
bb433
Host type
native
nonnative


(a) Host fitness exp.
(b) Symbiont transmission exp.
Infection prevalence
100
75
50
25
0
bb859
bb395
bb433
Symbiont strain
Host type
native
nonnative