Do Interactions among Microbial Symbionts Cause Selection for Greater Pathogen Virulence?

Georgiana May,1,* Ruth G. Shaw,1 Charles J. Geyer,2 and Daniel J. Eck3

1. Department of Ecology, Evolution, and Behavior, University of Minnesota, St. Paul, Minnesota 55108; 2. School of Statistics, University of Minnesota, Minneapolis, Minnesota 55455; 3. Department of Statistics, University of Illinois, Champaign, Illinois 61820

Submitted January 19, 2021; Accepted June 23, 2021; Electronically published January 6, 2022

Online enhancements: supplemental PDF.

ABSTRACT: The ecological and evolutionary consequences of microbiome treatments aimed at protecting plants and animals against infectious disease are not well understood, even as such biological control measures become more common in agriculture and medicine. Notably, we lack information on the impacts of symbionts on pathogen fitness with which to project the consequences of competition for the evolution of virulence. To address this gap, we estimated fitness consequences for a common plant pathogen, Ustilago maydis, over differing virulence levels and when the host plant (Zea mays) is coinfected with a defensive symbiont (Fusarium verticillioides) and compared these fitness estimates to those obtained when the symbiont is absent. Here, virulence is measured as the reduction in the growth of the host caused by pathogen infection. Results of aster statistical models demonstrate that the defensive symbiont most negatively affects pathogen infection and that these effects propagate through subsequent stages of disease development to cause lower pathogen fitness across all virulence levels. Moreover, the virulence level at which pathogen fitness is maximal is higher in the presence of the defensive symbiont than in its absence. Thus, as expected from theory for multiple parasites, competition from the defensive symbiont may cause selection for increased pathogen virulence. More broadly, we consider that the evolutionary impacts of interactions between pathogens and microbial symbionts will depend critically on biological context and environment and that interactions among diverse microbial symbionts in spatially heterogeneous communities contribute to the maintenance of the highly diverse symbiotic functions observed in these communities.

Keywords: microbiome, defensive symbiosis, parasites, virulence evolution, disease control, aster analysis.

Introduction

A plethora of recent studies have forwarded the notion that diverse microbial symbionts, the microbiome, play a central and largely positive role in the health of animals (Bordenstein and Theis 2015) and plants (Porras-Alfaro and Bayman 2011). Although microbiome control of disease and pests is increasingly sought (Scheuring and Yu 2012; Keper et al. 2017; Schlatter et al. 2017; Syed Ab Rahman et al. 2018) and may slow the evolution of resistance to antibiotics (Sommer and Dantas 2011; Tosh and McDonald 2012) or reduce dependence on environmentally damaging chemicals (Xue et al. 2015), recent empirical and theoretical results provide a counterpoint to a view that microbes and their hosts necessarily coevolve to mutualistic states (Antwis et al. 2015; Jani and Briggs 2018; Stevens et al. 2021). Competitive interactions between symbionts (Hoesema and Kummel 2003; Ford and King 2016; Nelson and May 2020) and between parasites (Bell et al. 2006; Barrett et al. 2011; Alizon et al. 2013) may cause a shift to less beneficial communities (Johnson et al. 1997) and selection on greater parasite virulence (Moran and Sloan 2015; Nelson and May 2017). Certainly, the ecological and evolutionary consequences of the “tangled bank” of multiple microbial interactions within hosts are poorly understood but are important to sustainable use of defensive symbionts in disease control (Betts et al. 2016; Busby et al. 2016; Ford et al. 2016; King et al. 2016; Keskella et al. 2017; Toju et al. 2018; Stevens et al. 2021).

A key question is whether the competitive interactions among co-occurring symbionts within a host might have the immediate effect of strengthening directional selection on the virulence of symbionts and thereby increase the risk of nonbeneficial trait evolution. Most studies have assessed the impacts of multiple parasite infections and generated the broad consensus that competition among parasites should often lead to the evolution of greater virulence (Bell et al. 2006; Martinsen et al. 2008; Barrett et al. 2011; Alizon et al. 2013). With the realization that defensive symbionts may often be closely related to pathogens (Faeth 2002; Pan...
et al. 2008; Jaenike et al. 2010; Busby et al. 2016), that body of theory has been extended to show that evolutionary outcomes for virulence will depend on the relative costs and benefits of the defense and virulence and on standing genetic variation for those traits (Fenton et al. 2011; Ford et al. 2016; King et al. 2016; Nelson and May 2020). Although less well explored, virulence outcomes might also depend on the life histories of the microbes within the host (Gandon et al. 2001; Balmer et al. 2009; Rigaud et al. 2010; Bruns et al. 2014). Together, results of both empirical and theoretical studies illustrate two critical limitations to our understanding and use of defensive symbionts in disease control, raising the following questions: how does symbiont-mediated host defense affect the parasite’s life history in the host and interactions with the host, and under what conditions do symbiont-parasite interactions cause selection toward greater parasite virulence?

To address these questions, we assessed the effects of a defensive symbiont, *Fusarium verticillioides*, on fitness outcomes for the plant pathogen *Ustilago maydis* when these two fungi interact within a common plant host, *Zea mays*. The corn smut pathogen *U. maydis* is naturally occurring on maize, as it followed the host through domestication from teosinte (Munkacsi et al. 2008), and the ascomycete *F. verticillioides* occurs commonly on maize as an endophyte, an asymptomatic symbiont (Pan et al. 2008). Different strains of *F. verticillioides* exhibit a wide range of interactions with the host from parasite to defensive symbiont (Glenn et al. 2001; Lee et al. 2009). Here, in replicated three-way experiments, the interactions among pathogen, defensive symbiont, and host were assessed, and progress through pathogen life history stages from infection to spore production was evaluated with and without the defensive symbiont. We defined virulence quantitatively as the negative impact on host growth and survival caused by pathogen infection, consistent with the quantitative resistance of maize to *U. maydis* (Baumgarten et al. 2007). We used pathogens strains that had been separately determined to have genetically differing virulence levels (Lee 2010) and assessed the relationship between pathogen reproduction and virulence. To obtain estimates for lifetime fitness of the pathogen in the presence and absence of the defensive symbiont, we applied aster statistical analyses that account for the contingency of sequential pathogen life history stages (Bruns et al. 2014; Alexander et al. 2017) and the impact of the defensive symbiont on the pathogen’s development through these stages (Geyer et al. 2007; Shaw et al. 2008).

**Material and Methods**

**Experimental Design**

As described in detail below, replicated factorial treatment combinations were established in the host plant *Zea mays* by inoculating each of 24 *Ustilago maydis* genotypes (or none) and one of two strains of the defensive symbiont, *Fusarium verticillioides* (or none). Over a 30-day period after inoculation, we assessed plant growth and pathogen life history stages from infection to reproduction. A strength of this and other plant-microbial systems is that fungal strains can be clonally propagated and the maize line is an inbred uniform genotype, ensuring that treatment replications are also precisely genetically replicated.

The life cycle of the pathogen *U. maydis* is obligate on the host because infection and subsequent reproduction requires mating between two compatible haploid yeast-like cells (sporidia) on the surface of the plant, followed by growth of an infectious dikaryotic (two parental nuclei per cell) filamentous hypha into the plant tissue. Growth of *U. maydis* within the host is constrained to the area of the infection (not systemic throughout the host) and culminates in the production of a plant tumor (gall) filled with diploid teliospores (6–11 µm in diameter) and covered by a thin “membrane” of plant tissue (Durán 1987). Because *U. maydis* does not produce asexual spores in the plant and the teliospores constitute the only mechanism of reproduction from the host, we use gall dry weight as a proxy measure of pathogen fitness.

**Fungal and Plant Genotypes**

We generated haploid strains of *U. maydis* that when mated on the plant produced infectious dikaryons of differing levels of virulence toward the maize host (Lee 2010). In brief, the dikaryon genotype UM2-P was generated by mating two haploid strains (C7 [mating type a1 b12; northern Ohio] and E11 [mating type a2 b11; Owatanna, MN]), and it exhibited high levels of virulence. The UM2-P teliospores obtained from galls were germinated, whereupon they undergo meiosis and produce haploid sporidial cells. More than 250 haploid strains were isolated and tested for compatibility with the parental strain C7 (a1 b12), and from these 23 haploid strains (UM2-1 to UM2-23; a2 b11) were randomly chosen. When each of these 23 haploid strains or the parental haploid E11 were coincubated with the parental strain C7 on the plant to generate UM2-P and UM2-1 to UM2-23 dikaryotic genotypes, they demonstrated varied levels of virulence toward the susceptible maize host (variety Jubilee, *Z. mays* var. *rugosa*; Jordan seeds, Woodbury, MN; fig. A1, adapted from fig. 2.1 in Lee 2010). We used two strains of *F. verticillioides* (FV1 [49_56796-85] and FV2 [20_57001-7E]; University of Minnesota Living Fungal Culture Collection) that had previously been isolated from maize in St. Paul, Minnesota (Pan et al. 2008), and do not cause disease symptoms in maize (Lee et al. 2009).

Plant inoculations were conducted as described elsewhere (Lee et al. 2009). In brief, haploid *U. maydis* cells were
grown separately in potato dextrose broth (Difco) to a density of approximately $2 \times 10^6$ cells/mL, harvested by centrifugation, washed twice with sterile water, and resuspended in sterile water to a final concentration of $10^8$ cells/mL. Spores of each *F. verticillioides* strain were collected from 10–15-day cultures on potato dextrose agar, washed twice with sterile water, and resuspended in sterile water to a concentration of $10^8$ cells/mL. In UM-only treatment inoculations, $10^7$ cells of the two compatible *U. maydis* haploid strains were mixed and inoculated in 0.4 mL of water by pipetting into the leaf whorl of seedlings 10 days after planting with minimal damage to the plant. For *F. verticillioides* inoculations, $2 \times 10^5$ spores of either the FV1 strain or the FV2 strain in 0.4 mL of water were inoculated (FV1- or FV2-only treatment). In UM + FV treatments, both fungal species were coinoculated with the same spore numbers in 0.4 mL of water, as described above. Mock inoculations were with 0.4 mL of sterile water only. The complete factorial design yielded 25 UM treatment levels (24 UM genotypes plus no UM control) and three FV treatments (FV1, FV2, or no FV control), for a total of 75 treatment combinations. To avoid cross contamination and obtain high levels of replication, six plants per pot received the same treatment, and eight replicate pots per treatment were inoculated with a randomized complete block (two greenhouse benches) design. Eight-inch pots were filled with Sunshine Professional Growing Mix (Sun Gro Horticulture Canada), and six seeds, previously surface sterilized, were planted in each pot. Greenhouse conditions were 20°C–28°C and a 14L:10D photoperiod with light intensities at 120–200 μE/m², at the University of Minnesota Plant Growth Facilities (St. Paul, MN).

We used gall dry weight (GallWT) as a proxy measure of pathogen reproduction because at maturity, galls are filled with *U. maydis* teliospores, spore size is consistent for the species (6–11 μM), and there is very little plant material in galls (Durán 1987). Because even small galls contain far too many spores to count, we previously had estimated the relationship between gall weight and spore count at $3.5 \times 10^2 \pm 4.2 \times 10^1$ (SE) spores/mg gall dry weight for a wide range of gall weights by subsampling small dry weights, diluting these, and counting spores in small volumes using a hemocytometer (Lee 2010). In addition, because the host is an annual plant and *U. maydis* does not produce asexual spores from the host, as do some plant pathogens, teliospore production represents the only pathway of reproduction from the host plant under both greenhouse and field conditions.

The mean virulence attributed to each *U. maydis* dikaryotic genotype was measured as the average decrease in plant growth caused by inoculation with that genotype relative to the mean growth of control plants without *U. maydis* inoculation (fig. A1, adapted from Lee 2010). Our quantitative measure of virulence is appropriate to the multigenic quantitative resistance of maize to *U. maydis* described previously (Baumgarten et al. 2007) and is distinct from gene-for-gene systems in which pathogen infection is determined by the interaction of discrete pathogen virulence (*Avir*) genes with specific resistance (*R*) genes in plants (Flor 1971). The mean virulence attributed to each *U. maydis* genotype is comparable to the term “intrinsic virulence” used for pathogens of animals (e.g., Gandon et al. 2001).

**Evaluation of Pathogen Life History Stages, Fitness, and Virulence**

Life history stages were assessed visually to avoid destructive sampling and allow assessment of lifetime fitness of the pathogen in individual plants. We assessed pathogen life history stages 10 and 30 days postinoculation (hereafter, DPI10 and DPI30) using qualitative scores similar to those of Klosterman et al. (2007). Individual plants with no visible symptoms of infection by *U. maydis* were scored as 0; infection was observed as the presence of anthocyanin and/or leaf or stem chlorosis and was scored as 1; the appearance of small developing leaf galls was scored as 2; the presence of small developing stem galls was scored as 3; the presence of fully developed galls filled with teliospores was scored as 4; and plant death was scored as 5. All plants were scored blind without reference to treatments. The experiment was terminated at DPI30, and the mature galls with black teliospores were carefully excised, collected together from each individual plant, and dried at 60°C to constant dry weight. We used aster analyses with dependency groups (Eck et al. 2015a, 2015b) to estimate the effects of experimental variables (*Invir*, the mean “intrinsic” virulence of each pathogen genotype, and *Fvert*, the absence or presence of either defensive symbiont strain, FV1 or FV2) on pathogen fitness (GallWT). The aster approach accounts for the contingency of sequential life history stages (i.e., successful infection must precede pathogen growth in the host and reproduction). Thus, our aster estimates of mean fitness (as spore production) take into account transitions at preceding life history stages, including pathogen infection and continuing growth (or not) within the host as well as the differing error distributions that may be associated with each transition. Parameters in the final model correspond to overall fitness. The life history transitions and dependencies are represented in the aster graph (Geyer et al. 2007; Shaw et al. 2007, 2008) and were obtained in this work as described next.

For each individual plant, we used the disease progress scores described above and additional observations to

**Aster Analysis**

We used aster analyses with dependency groups (Eck et al. 2015a, 2015b) to estimate the effects of experimental variables (*Invir*, the mean “intrinsic” virulence of each pathogen genotype, and *Fvert*, the absence or presence of either defensive symbiont strain, FV1 or FV2) on pathogen fitness (GallWT). The aster approach accounts for the contingency of sequential life history stages (i.e., successful infection must precede pathogen growth in the host and reproduction). Thus, our aster estimates of mean fitness (as spore production) take into account transitions at preceding life history stages, including pathogen infection and continuing growth (or not) within the host as well as the differing error distributions that may be associated with each transition. Parameters in the final model correspond to overall fitness. The life history transitions and dependencies are represented in the aster graph (Geyer et al. 2007; Shaw et al. 2007, 2008) and were obtained in this work as described next.

For each individual plant, we used the disease progress scores described above and additional observations to
determine pathogen developmental life history stages as follows: at DPI10, infection by *U. maydis* or no infection (score of 1 or 0; node I); at DPI10 or DPI30, infection persisted or not (score of 2 or 3 vs. no score; node per); and at DPI30, *U. maydis* reproduction (dry weight of mature galls per plant; node GallWT). Infection is a binary 0, 1 event and is modeled with a Bernoulli distribution. Because pathogen infections may occur in the leaves or the stem (and rarely both), we designated separate leaf (score 2, LH2) and stem (score 3, LH3) paths in the aster analysis. These LH2 and LH3 paths, along with LH1 (no further development), form a dependency group that is modeled with a multinomial distribution. The multinomial distribution reflects a trifurcation where a particular plant can transition into only one of these three nodes. Persistence (per) is a 0, 1 event and modeled with a Bernoulli distribution. Last, to account for possible effects of plant death on disease development and reproduction, we incorporated a node for plant dies (PD0) or plant lives (PD1) after the persistence (pers) node on each of the leaf and stem developmental paths. Plant death is modeled with a multinomial distribution where PD0 and PD1 form a dependency group. In this study, plants with leaf infections alone (no stem galls) did not die. Finally, GallWT is modeled with a normal distribution (fig. 1).

We used backward model selection starting with the complete model that included linear effects for greenhouse bench (Block), *F. verticillioides* treatment (Fvert), *U. maydis* genotypic virulence level (Invir), a quadratic effect for *U. maydis* virulence (Invir²), and interaction terms between Fvert and Invir and between Fvert and Invir² for both the infection (I) and gall weight (GallWT) nodes (see fig. 1; table A1). The complete aster model is designated as follows (nodes in boldface): resp ~ varb + I(Block + Fvert + Invir + I(Invir²) + Fvert * Invir + Fvert * I(Invir²) + I(Fvert: Block)) + GallWT:(Block + Fvert + Invir + I(Invir²) + Fvert * Invir + Fvert * I(Invir²) + I(Fvert:Block)). For comparison to the results of the above complete model, we also evaluated a GallWT aster model in which the effects of the symbiont and virulence are evaluated at the GallWT node: resp ~ varb + GallWT:(Block + Fvert + Invir + I(Invir²) + Fvert * Invir + Fvert * I(Invir²) + I(Fvert:Block)). In aster models, the effects on the distribution of fitnesses at the last node (here, GallWT) propagate backward to predecessor nodes by affecting the distributions of earlier expressed components of fitness (Shaw et al. 2008).

**Generalized Linear Model (GLM) Analysis**

Separately from the above-described aster analyses, we used a GLM to model the probability of infection as a function of the mean virulence attributed to each pathogen genotype (Invir) and the absence or presence of either of two strains of the defensive symbiont (Fvert). The full model was Infect ~ Invir * Fvert + I(Invir²), with infection (Infect) as a 0, 1 observation for each individual plant inoculated with a single *U. maydis* genotype. The significance of terms in the logistic regression were evaluated with a binomial error distribution (R function glm; R Core Team 2020) using backward model selection (tables A3, A4).

---

**Figure 1:** Aster graph. Pathogen life history paths are designated LH. After infection (I), an individual plant may exhibit no further disease development (LH1), development on leaves (LH2), or development on stems (LH3); thus, LH1, LH2, and LH3 form a dependency group. If the pathogen growth in the plant persists (per) after infection, it may either cause plant death (PD0) or not (PD1). Since individual plants either live or die but not both, PD0 and PD1 form a dependency group within each developmental path, LH2 or LH3. For both paths, the pathogen may produce galls filled with spores, a measure of reproduction (GallWT). In the LH3 path, stem galls were produced whether the plant lived (PD1) or died (PD0), whereas in the LH2 path, plant death did not occur.
Results

When inoculated individually and in the absence of the defensive symbiont, the 24 Ustilago maydis dikaryotic genotypes demonstrate continuous variation in mean virulence (decreased host plant height growth compared with that for noninoculated control plants). The parental genotype UM2-P exhibited an intermediate level of virulence (mean height loss of 20.8 ± 1.9 [SD] cm), dikaryotic genotype UM2-3 exhibited the highest level of virulence (mean height loss of 31.2 ± 0.7 [SD] cm), and dikaryotic genotype UM2-23 exhibited the lowest level of virulence and caused little loss of plant growth (mean height loss of 0.23 exhibited the lowest level of virulence and caused little loss of plant growth (mean height loss of 0.8 ± 1.2 [SD] cm) and no visible galls (fig. A1). Four plants had died at DPI30, all of which were inoculated with U. maydis strains of high virulence levels. We estimated that >90% of all plants inoculated with Fusarium verticillioides harbored the defensive symbiont, as evidenced by reisolation of F. verticillioides from plant tissues and the presence of F. verticillioides spores in U. maydis galls. Consistent with previous observations (Lee et al. 2009), F. verticillioides strains FV1 and FV2 did not cause visible disease symptoms in control inoculations of either strain alone in plants; instead, mean growth of plants inoculated with the defensive symbiont alone was 2%–3% greater than that in noninoculated control plants (control: 58.4 ± 8.2 [SD] cm; FV1: 60.2 ± 8.3 [SD] cm; FV2: 59.8 ± 9.4 [SD] cm). None of the noninoculated control plants were infected with either U. maydis or F. verticillioides, evidence of very low plant-to-plant transmission in the greenhouse conditions.

Aster Analyses

As described above, the complete aster model included linear effects for greenhouse bench (Block), F. verticillioides treatment (Fvert), U. maydis genotypic virulence level (Invir), a quadratic effect for U. maydis virulence (Invir²), and interaction terms between Fvert and Invir and between Fvert and Invir² for both the infection (I) and gall weight (GallWT) nodes (fig. 1). In the backward model selection, the contribution of each predictor for each node (infection or GallWT) is evaluated sequentially (table A1). The result of model selection gave the following: resp ~ varb + I:(Block + Fvert + Invir) + GallWT:(Block + Invir + I(Invir²)). Three attributes of the complete model with infection and GallWT nodes are notable: (1) infection is best predicted by the linear term for virulence (Invir), while pathogen reproduction (GallWT) is better predicted by including both the linear and the quadratic terms for virulence (Invir²); (2) infection, but not pathogen reproduction (GallWT), includes the term for the absence or presence of either defensive symbiont (FV1 or FV2); and (3) the interaction term for the defensive symbiont strain (or none) and genotypic virulence level of the pathogen (Fvert × Invir) did not contribute significantly to the model for either the infection node or the GallWT node (table A1).

Using aster2 software, we estimated expected pathogen fitness (spore mass, in grams) for the cases in which the defensive symbiont is absent or present (strain FV1 or strain FV2) and over a range of virulence levels consistent with the experimental values. The results demonstrate that the defensive symbiont reduces the pathogen expected fitness across all virulence levels and that inoculation with strain FV1 has a stronger negative impact on pathogen fitness than does inoculation with strain FV2 (fig. 2A). The maximal estimated pathogen fitness in the absence of the defensive symbiont is estimated at 9.4 × 10⁻² g of dry weight (95% confidence interval [CI]: 9.0 × 10⁻² to 9.8 × 10⁻² g). Compared with the estimated pathogen fitness in the absence of the defensive symbiont, estimated pathogen fitness is 16.8% lower (7.8 × 10⁻² g; 95% CI: 7.4 × 10⁻² to 8.1 × 10⁻²) in the presence of the defensive symbiont strain FV1 and 13.8% lower (8.1 × 10⁻² g; 95% CI: 7.7 × 10⁻² to 8.5 × 10⁻²) in the presence of defensive symbiont strain FV2. At the highest virulence levels expected pathogen fitness declines, regardless of the defensive symbiont treatment, as a greater number of plant deaths occurs and plant growth is ~50% less than controls. Last, when either defensive symbiont strain is present, the expected pathogen fitness is maximal at a virulence level that is ~7% higher (loss of 27.9 cm of plant height) than when the defensive symbiont is absent (loss of 26.1 cm of plant height).

In evaluating the complete aster model, we found that the term for the defensive symbiont contributed significantly at the infection (I) node but not at the GallWT node (fig. 1). To further investigate the consequences of the defensive symbiont for expected pathogen fitness within the aster framework, we analyzed a second, more limited aster model that accounted for the impact of virulence and the defensive symbiont at pathogen reproduction nodes (GallWT) only and compared those results with those for the complete aster model given above. Terms for the defensive symbiont treatment (Fvert) and the linear and quadratic virulence terms were retained, but the Block and interaction terms were not (table A2), to give the following final model: resp ~ varb + GallWT:(Fvert + Invir + I(Invir²)). We obtained values of expected pathogen fitness of the GallWT model using functions of aster2 software as described above. Results are similar to those obtained in the complete model except that the virulence level (loss of 28.6 cm of plant growth) at which pathogen fitness is maximal is the same in the presence or absence of the defensive symbiont (fig. 2B).

GLM Analysis

Results of the complete aster model (table A1; fig. 2A) show that the defensive symbiont had the strongest impact on
pathogen fitness through its effect on infection, the first stage of disease development, and that the two strains of *F. verticillioides* had somewhat different effects. To evaluate the impacts of the defensive symbiont on pathogen infection, we used a separate GLM analysis of the probability of pathogen infection given the virulence level attributed to each *U. maydis* genotype and in the presence or absence of either defensive symbiont strain. Results of model selection retained the factor for the defensive symbiont treatment (Fvert; none, FV1, or FV2) and for the linear and quadratic terms for *U. maydis* virulence (Invir, Invir^2) but not for the Fvert × Invir interaction term (table A3). These results show that the probability of infection increases with greater *U. maydis* virulence levels and further suggest that inoculation with strain FV1 is associated with a greater reduction in pathogen infection rates than is strain FV2 (fig. 3), although the effects of the two strains on pathogen infection (table A4) are not greatly different, especially at higher virulence levels.

Together, the aster results show evidence of a trade-off as expected pathogen fitness declines at the highest virulence
control and, more broadly, for the maintenance of diversity in microbiome symbiont communities.

Given that pathogen virulence affects host growth, the trade-off of pathogen virulence and reproduction we observe is consistent with previous theoretical and empirical results for parasite-host interactions in which costs of virulence limit the resources for pathogen growth or reproduction within the host (Anderson and May 1978; Messenger et al. 1999; de Roode et al. 2008; Alizon et al. 2009). In this pathogen-host interaction, that cost is likely the subversion of host photosynthetic carbon sources away from plant growth to the sink of pathogen growth and development (Doehlemann et al. 2008) and the earlier death of plants with *Ustilago maydis* infections (Lee et al. 2009). Remarkably, maximum pathogen reproduction was obtained at virulence levels that cause a loss of half the potential plant height growth, implying an efficient transfer of photosynthetic resources to the pathogen. Because we do not find evidence for statistical interaction terms of virulence and the presence or absence of either defensive symbiont strain, our results further suggest direct interactions between the defensive symbiont and the pathogen at the infection stage rather than indirect effects, such as the symbiont affecting host immunity responses (Conn et al. 2008; Torto-Alalibo et al. 2009; Gourion et al. 2015; Kim et al. 2015).

Competition among parasites can cause selection on virulence (e.g., van Baalen and Sabelis 1995; de Roode et al. 2005; Alizon et al. 2013), but outcomes also depend on the life history stages at which parasites interact (Bell et al. 2006; Rutrecht and Brown 2008; Balmer et al. 2009; Rigaud et al. 2010). Here, results from the aster analysis show that the defensive symbiont, *Fusarium verticillioides*, most strongly affects pathogen infection possibly via production of numerous secondary compounds (Rodriguez-Estrada et al. 2012; Jonkers et al. 2012) that may impede pathogen mating on the plant surface. That negative impact on infection by the defensive symbiont propagates through the pathogen life history in the host with two apparent consequences: an overall loss of 14%–17% of pathogen fitness, and an upward ∼7% shift of virulence levels at which pathogen reproduction is maximal. Thus, competition between a defensive symbiont and parasite can cause selection toward greater pathogen virulence in much the same way as competition between parasites (Bell et al. 2006; Martinsen et al. 2008; Barrett et al. 2011; Alizon et al. 2013).

Competition among parasites can cause selection on virulence (e.g., van Baalen and Sabelis 1995; de Roode et al. 2005; Alizon et al. 2013), but outcomes also depend on the life history stages at which parasites interact (Bell et al. 2006; Rutrecht and Brown 2008; Balmer et al. 2009; Rigaud et al. 2010). Here, results from the aster analysis show that the defensive symbiont, *Fusarium verticillioides*, most strongly affects pathogen infection possibly via production of numerous secondary compounds (Rodriguez-Estrada et al. 2012; Jonkers et al. 2012) that may impede pathogen mating on the plant surface. That negative impact on infection by the defensive symbiont propagates through the pathogen life history in the host with two apparent consequences: an overall loss of 14%–17% of pathogen fitness, and an upward ∼7% shift of virulence levels at which pathogen reproduction is maximal. Thus, competition between a defensive symbiont and parasite can cause selection toward greater pathogen virulence in much the same way as competition between parasites (Bell et al. 2006; Martinsen et al. 2008; Barrett et al. 2011; Alizon et al. 2013).

Considering demographic and genetic effects together, we address the question of whether deployment of defensive symbionts might limit pathogen spread but have the unintended consequence of selection on greater pathogen virulence. Our greenhouse experiments imposed conditions by which we could best observe the impacts of the defensive symbiont on the fitness of the pathogen—the pathogen and defensive symbiont co-occurred at a high rate in...
coinoculated plants, and environmental conditions were consistent across individual plants. These empirical results are thus most informative to situations that might be obtained in agricultural or other human-managed systems in which biological agents are deployed to control pests or pathogens. For example, biological control measures that are either applied in high-dose treatments or engineered into plants to obtain uniform expression and deployed over host populations without susceptible plant refugia may lead to evolution of pest resistance to control measures (Alstad and Andow 1995; Andow and Ives 2002). Such undesired outcomes may be obtained either by ecological processes, such as the displacement of mutualistic symbionts by more strongly competing, less beneficial symbionts (Johnson et al. 1997), or by the rapid evolution of traits less beneficial to the host (Weese et al. 2015). In contrast, symbionts such as *F. verticillioides* that lower pathogen infection may act as a “vaccine” and reduce the prevalence of the pathogen (Gandon et al. 2001) to a level at which stochastic processes dominate coevolutionary interactions (André and Hochberg 2005). Together, our results illustrate that the joint estimation of demographic and genetic effects available through aster analyses will improve predictions for the trajectories of the interrelated processes of pathogen spread and virulence evolution.

More broadly, understanding the impacts of co-occurring symbionts on the evolution of parasite virulence informs the underlying principles of symbiotic community assembly and the sustainable deployment of biological control measures. In particular, while deeply diverse microbiomes are often observed in natural and human-managed systems (Pan et al. 2008; Antwis et al. 2015; van der Heijden and Hartmann 2016; Schlatter et al. 2017; U’Ren et al. 2019), the evolutionary processes that generate and maintain genetic variation in ecological function of symbiont populations are far less well understood (Oliver et al. 2005; Castro-Sowinski et al. 2007; Coyte et al. 2015; Lewis et al. 2019). Although limited to a three-way interaction, the results of our experimental study support hypotheses that weak and varied competition among symbionts reduces the mean strength of directional selection (Gomulkiewicz et al. 2000; Laine 2009; Coyte et al. 2015) and promotes the coexistence of parasitic, commensal, and mutualistic symbiont communities (Van Dyken et al. 2013; Liu et al. 2014; Coyte et al. 2015). If so, identifying symbionts with moderate costs of defense (Nelson and May 2020) and establishing these control agents within diverse communities (Dattilo et al. 2016; Kepler et al. 2017; Syed Ab Rahman et al. 2018; Toju et al. 2018; Vonaesch et al. 2018) that interfere with pathogens throughout their life history within the host should reduce the risk of escalating virulence and slow emergence of new infectious disease (Smith et al. 2002), as does heterogeneity in host populations (Zhu et al. 2000; Andow and Ives 2002) and use of multiple drug treatments (e.g., Simon et al. 2006).

### Conclusions

In this work, we examine the joint demographic and evolutionary impacts of interactions between a defensive symbiont and a pathogen within the host. Using aster statistical analyses, we show that competition between the defensive symbiont and pathogen is strongest at the host infection stage of the life history and propagates through the pathogen life history to cause decreased pathogen lifetime fitness. Our results further predict selection on pathogen virulence, similar to that expected from theory for interacting pathogens within hosts. Together, the results of this and other studies broaden our understanding of the ecological and evolutionary impacts of interacting microbial symbionts within hosts and inform strategies for use of defensive symbionts in host protection. In conclusion, we suggest that experimental and theoretical analyses that recognize the functional diversity of symbionts along a continuum from mutualism to parasitism will yield the greater insight into the ecological and evolutionary processes affecting symbiont-host population dynamics (Akhhami et al. 2014; May 2016; Lemos-Costa et al. 2017; Nelson and May 2020; Stevens et al. 2021).

### Acknowledgments

We thank Keunsub Lee for tireless and careful work in his dissertation research (partially supported by National Science Foundation [NSF] grant DEB-0723451 to G.M.). The aster analyses presented here were supported by NSF grant DEB-1046065 to G.M. and were conducted by coauthors D.J.E. and C.J.G. We thank two anonymous reviewers for thoughtful and constructive comments and American Naturalist editors for careful consideration of the manuscript and constructive suggestions and edits. G.M. and R.G.S. acknowledge continuing inspiration of the University of Minnesota Center for Community Genetics.

### Statement of Authorship

G.M. conceived of the project, guided data collection, obtained funding, developed the aster graph with D.J.E., and wrote the original draft. R.G.S. advised application of aster models and provided feedback on statistical models and interpretation of results. C.J.G. provided statistical conceptualization of aster2 analyses, aster2 software development, and feedback on interpretation of results. D.J.E. provided statistical conceptualization and programming, developed the aster graph with G.M., ran aster2 analyses, and gave feedback on interpretation of results. All coauthors read and provided feedback and edits on the original draft of the manuscript and all subsequent revisions.
APPENDIX

Figure A1: Mean virulence of pathogen genotypes. Virulence is measured as the mean loss of plant height due to inoculation with each of the 24 different *Ustilago maydis* genotypes (x-axis) relative to the mean height of the control plants not inoculated with *U. maydis* (mock inoculated with water). The virulence measure included all plants inoculated with a given *U. maydis* genotype. The standard error for each mean is shown and was calculated from the pooled standard deviation of control and pathogen-inoculated plants. *Ustilago maydis* genotypes are ordered from lowest to greatest virulence, and the parent genotype, U2-P, is indicated by the gray arrow. Data from Lee (2010).
Table A1: Backward model selection model in aster for the complete model accounting for effects of *Ustilago maydis* mean genotypic virulence (Invir) and the presence or absence of the defensive symbiont, *Fusarium verticillioides* (Fvert; strain FV1, strain FV2, or none), at both the infection (I) node and the gall weight (GallWT) node (see fig. 1)

| Model      | Infection node terms | Gall weight node terms                  | P     |
|------------|----------------------|-----------------------------------------|-------|
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | 1.458 × 10⁻⁵ |
| Alt        | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | .014  |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | .74   |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | .053  |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | 1.14 × 10⁻⁶ |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | .92   |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | ~0    |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | .85   |

Note: At each step, for each node the "null" model drops one term of the larger "alt" model (in boldface) and compares the fit of the models using a Rao test statistic at α = .05 (Eck et al. 2015b). In the complete model, the terms Fvert and Invir remain significant for the infection node, whereas Invir and Invir2, but not Fvert, are significant for the GallWT node. Block (greenhouse bench) remains in the model for both nodes. Results for expected pathogen fitness using the complete model are shown in figure 2A.

Table A2: Backward model selection model in aster for the GallWT model accounting for effects of *Ustilago maydis* mean genotypic virulence (Invir) and the presence or absence of the defensive symbiont, *Fusarium verticillioides* (Fvert; strain FV1, strain FV2 or none) at the gall weight (GallWT) node only (see fig. 1)

| Model      | Gall weight node terms                  | P     |
|------------|-----------------------------------------|-------|
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | .92   |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | .64   |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | .16   |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | 2.56 × 10⁻⁴ |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | 2.4 × 10⁻⁴ |
| Null       | Block, Fvert, Invir, Invir2, Fvert × Invir, Fvert × Invir2 | .46   |

Note: At each step, the "null" model drops one term of the larger "alt" model (in boldface) and compares the fit of the two models using a Rao test statistic at α = .05 (Eck et al. 2015b). In the final GallWT model, Fvert, Invir, and Invir2 remain, and Block (greenhouse bench) and all interaction terms are not retained. Results for expected pathogen fitness using the GallWT model are shown in figure 2B.
Table A3: Results of backward selection for the generalized linear model of infection and virulence

|                | Estimate | SE   | z     | Pr(>|z|) |
|----------------|----------|------|-------|----------|
| Intercept      | -1.75174 | .222183 | -7.884  | 3.16 × 10^{-15}*** |
| Invir          | .181691  | .023885 | 7.607  | 2.81 × 10^{-14}*** |
| Fvert          | -.320511 | .048197 | -6.65  | 2.93 × 10^{-11}*** |
| I(Invir^2)     | -.002227 | .000629 | -3.537 | .000404*** |

Note: The full model for infection (Infect; 0 or 1) included the linear and quadratic terms for virulence of each Ustilago maydis genotype (Invir, Invir^2); the term for the absence of the defensive symbiont, the presence of strain FV1, or the presence of strain FV2 (Fvert); and the interaction term (Infect ~ Invir × Fvert + I(Invir^2), family = binomial (link = "logit"). The Invir × Fvert interaction term is dropped from the model (Pr(>|z|) = 0.9598), and the reduced model evaluated (Infect ~ Invir + Fvert + I(Invir^2), family = binomial (link = "logit"). Results are presented in figure 3. Akaike information criterion: 3,882.9.

*** P < .001.

Table A4: Results of backward selection for the generalized linear model of infection and virulence in which the factor Fvert01, for the absence of the defensive symbiont or the presence of either strain (FV1 or FV2), is included in place of the Fvert term in table A3

|                | Estimate | SE   | z     | Pr(>|z|) |
|----------------|----------|------|-------|----------|
| Intercept      | -1.601072 | .224546 | -7.13  | 1.00 × 10^{-12}*** |
| Invir          | .184096  | .0240133 | 7.656  | 1.77 × 10^{-14}*** |
| Fvert01        | -.739877 | .0877252 | -8.434 | <2 × 10^{-16}*** |
| I(Invir^2)     | -.002264 | .0006323 | -3.581 | .000342*** |

Note: In the final model, the interaction term (Invir × Fvert01) is dropped. The model fit gives a slightly lower Akaike information criterion value (3,852.7) than in table A3, suggesting that the effects of the two strains are not greatly different.

*** P < .001.

Literature Cited

Afkhami, M. E., J. A. Rudgers, and J. J. Stachowicz. 2014. Multiple mutualist effects: conflict and synergy in multispecies mutualisms. Ecology 95:833–844.
Alexander, H. M., E. Bruns, H. Schebor, C. M. Malmstrom, and A. Power. 2017. Crop-associated virus infection in a native perennial grass: reduction in plant fitness and dynamic patterns of virus detection. Journal of Ecology 105:1021–1031.
Alizon, S., J. C. de Roode, and Y. Michalakis. 2013. Multiple infections and the evolution of virulence. Ecology Letters 16:556–567.
Alizon, S., A. Hurford, N. Mideo, and M. van Baalen. 2009. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. Journal of Evolutionary Biology 22:245–259.
Alstad, D. N., and D. A. Andow. 1995. Managing the evolution of insect resistance to transgenic plants. Science 268:1894–1896.
Anderson, R. M., and R. M. May. 1978. Regulation and stability of host-parasite population interactions: I. Regulatory processes. Journal of Animal Ecology 47:219–247.
Andow, D. A., and A. R. Ives. 2002. Monitoring and adaptive resistance management. Ecological Applications 12:1378–1390.
Andrè, J.-P., and M. E. Hochberg. 2005. Virulence evolution in emerging infectious diseases. Evolution 59:1406–1412.
Antwis, R. E., R. F. Preziosi, X. A. Harrison, and T. W. Garner. 2015. Amphibian symbiotic bacteria do not show a universal ability to inhibit growth of the global panzootic lineage of Batrachochytrium dendrobatidis. Applied and Environmental Microbiology 81:3706–3711.
Baumgarten, A. M., J. Suresh, G. May, and R. L. Phillips. 2007. Mapping QTLs contributing to Ustilago maydis resistance in specific plant tissues of maize. Theoretical and Applied Genetics 114:1229–1238.
Bell, A. S., J. C. de Roode, D. Sim, and A. F. Read. 2006. Within-host competition in genetically diverse malaria infections: parasite virulence and competitive interactions. Evolution 60:1358–1371.
Betts, A., C. Rafaluk, and K. C. King. 2016. Host and parasite evolution in a tangled bank. Trends in Parasitology 32:863–873.
Bordenstein, S. R., and K. R. Theis. 2015. Host biology in light of the microbiome: ten principles of holobionts and hologenomes. PLoS Biology 13:e1002226.
Bruns, E., M. L. Carson, and G. May. 2014. The jack of all trades is master of none: a pathogen’s ability to infect a greater number of host genotypes comes at a cost of delayed reproduction. Evolution 68:2453–2466.

Busby, P. E., K. G. Peay, and G. Newcombe. 2016. Common foliar fungi of Populus trichocarpa modify Melampsora rust disease severity. New Phytologist 209:1681–1692.

Castro-Sowinski, S., Y. Herschkovitz, Y. Okon, and E. Jurkevitch. 2007. Effects of inoculation with plant growth-promoting rhizobacteria on resident rhizosphere microorganisms. FEMS Microbiology Letters 276:1–11.

Conn, V. M., A. R. Walker, and C. M. M. Franco. 2008. Endophytic actinobacteria induce defense pathways in Arabidopsis thaliana. Molecular Plant-Microbe Interactions 21:208–218.

Coyte, K. Z., J. Schluter, and K. R. Foster. 2015. The ecology of the microbiome: networks, competition, and stability. Science 350:663–666.

Dattilo, W., N. Lara-Rodriguez, P. Jordano, P. R. Guimaraes Jr., J. M. Rangel, L. P. Medeiros, et al. 2016. Unravelling Darwin’s entangled bank: architecture and robustness of mutualistic networks with multiple interaction types. Proceedings of the Royal Society B 283:20161564.

DEck13. 2021. DEck13/aster_symbionts: first release of symbiotic data (v0.1). Zenodo, https://doi.org/10.5281/zenodo.5247017.

de Roode, J. C., M. E. H. Helsinki, M. A. Anwar, and A. F. Read. 2005. Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. American Naturalist 166:531–542.

de Roode, J. C., A. J. Yates, and S. Altizer. 2008. Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. Proceedings of the National Academy of Sciences of the USA 105:7489–7494.

Doehlemann, G., R. Wahl, R. J. Horst, L. M. Voll, B. Usadel, F. Poree, M. Stitt, et al. 2008. Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph Ustilago maydis. Plant Journal 56:181–195.

Durán, R. 1987. Ustilaginales of Mexico: taxonomy, symptomatology, spore germination, and basidial cytology. Washington State University Press, Pullman.

Eck, D. J., R. G. Shaw, C. J. Geyer, and J. G. Kingsolver. 2015a. An integrated analysis of phenotypic selection on insect body size and development time. Evolution 69:2525–2532.

———. 2015b. Supporting data analysis for “An integrated analysis of phenotypic selection on insect body size and development time.” Technical report. School of Statistics, University of Minnesota. https://conservancy.umn.edu/handle/11299/172272.

Faeth, S. H. 2002. Are endophytic fungi defensive plant mutualists? Oikos 98:25–36.

Fenton, A., K. N. Johnson, J. C. Brownlie, and G. D. Hurst. 2011. Solving the Wolbachia paradox: modeling the tripartite interaction between host, Wolbachia, and a natural enemy. American Naturalist 178:333–342.

Flor, H. 1971. Current status of gene-for-gene concept. Annual Review of Phytopathology 9:275–296.

Ford, S. A., D. Kao, D. Williams, and K. C. King. 2016. Microbe-mediated host defence drives the evolution of reduced pathogen virulence. Nature Communications 7:13430.

Ford, S. A., and K. C. King. 2016. Harnessing the power of defensive microbes: evolutionary implications in nature and disease control. PLoS Pathogens 12:e1005465.

Gandon, S., M. J. Mackinnon, S. Nee, and A. F. Read. 2001. Imperfect vaccines and the evolution of pathogen virulence. Nature 414:751–755.

Geyer, C. J. 2010. R package aster2 (aster models), version 0.1-1. http://cran.r-project.org/package=aster2.

———. 2014. R package aster (aster models), version 0.8-30. http://cran.r-project.org/package=aster.

Geyer, C. J., S. Wagenius, and R. G. Shaw. 2007. Aster models for life history analysis. Biometrika 94:415–426.

Glenn, A. E., D. M. Hinton, I. E. Yates, and C. W. Bacon. 2001. Detoxification of corn antimicrobial compounds as the basis for isolating Fusarium verticillioides and some other Fusarium species from corn. Applied and Environmental Microbiology 67:2973–2981.

Gomulkiewicz, R., J. N. Thompson, R. D. Holt, S. L. Niuism, and M. E. Hochberg. 2000. Hot spots, cold spots, and the geographic mosaic theory of coevolution. American Naturalist 156:156–174.

Gourion, B., F. Berrabah, P. Ratet, and G. Stacey. 2015. Rhizobium-legume symbioses: the crucial role of plant immunity. Trends in Plant Science 20:186–194.

Harris, R. N., R. M. Brucker, J. B. Walke, M. H. Becker, C. R. Schwantes, D. C. Flaherty, B. A. Lam, et al. 2009. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME Journal 3:818–824.

Hoeksema, J. D., and M. Kummel. 2003. Ecological persistence of the plant-mycorrhizal mutualism: a hypothesis from species coexistence theory. American Naturalist 162(suppl.):S40–S50.

Jaenike, J., R. Unckless, S. N. Cockburn, L. M. Boelio, and S. J. Perlman. 2010. Adaptation via symbiosis: recent spread of a Drosophila defensive symbiont. Science 329:212–215.

Jani, A. J., and C. J. Briggs. 2018. Host and aquatic environment shape the amphibian skin microbiome but effects on downstream resistance to the pathogen Batrachochytrium dendrobatidis are variable. Frontiers in Microbiology 9:487.

Johnson, N., J. H. Graham, and F. Smith. 1997. Functioning of mycorrhizal associations along the mutualism–parasitism continuum. New Phytologist 135:575–585.

Jonkers, W., A. E. Rodriguez Estrada, K. Lee, A. Breakspear, G. May, and H. C. Kistler. 2012. Metabolome and transcriptome of the interaction between Ustilago maydis and Fusarium verticillioides in vitro. Applied and Environmental Microbiology 78:3656–3667.

Kepler, R. M., J. E. Maul, and S. A. Rehner. 2017. Managing the plant microbiome for biocontrol fungi: examples from Hypocreales. Current Opinion in Microbiology 37:48–53.

Kim, J. K., J. B. Lee, Y. R. Huh, H. A. Jang, C.-H. Kim, J. W. Yoo, and B. L. Lee. 2015. Burkholderia gut symbionts enhance the innate immunity of host Riptortus pedestris. Developmental and Comparative Immunology 53:265–269.

King, K. C., M. A. Brockhurst, O. Vasieva, S. Paterson, A. Betts, S. A. Ford, C. L. Frost, et al. 2016. Rapid evolution of microbe-mediated protection against pathogens in a worm host. ISME Journal 10:1915–1924.

Klosterman, S. J., M. H. Perlin, M. Garcia-Pedrajas, S. F. Covert, and S. E. Gold. 2007. Genetics of morphogenesis and pathogenic development of Ustilago maydis. Advances in Genetics 57:1–47.

Koskella, B., L. J. Hall, and C. J. E. Metcalf. 2017. The microbiome beyond the horizon of ecological and evolutionary theory. Nature Ecology and Evolution 1:1606–1615.
Laine, A. L. 2009. Role of coevolution in generating biological diversity: spatially divergent selection trajectories. Journal of Experimental Botany 60:2957–2970.

Lee, K. 2010. The effects of endophytic *Fusarium verticillioides* on the interactions of maize and its fungal pathogen *Ustilago maydis*. PhD diss. University of Minnesota, conservancy.umn.edu/handle/11299/97147.

Lee, K., J. J. Pan, and G. May. 2009. Endophytic *Fusarium verticillioides* reduces disease severity caused by *Ustilago maydis* on maize. FEMS Microbiology Letters 299:31–37.

Lemos-Costa, P., A. B. Martins, J. N. Thompson, and M. A. M. de Aguilar. 2017. Gene flow and metacommunity arrangement affects coevolutionary dynamics at the mutualism-antagonism interface. Journal of the Royal Society Interface 14:20160989.

Lewis, M. H., I. Carbone, J. M. Luis, G. A. Payne, K. L. Bowen, A. K. Hagan, R. Kemerait, R. Heiniger, and P. S. Ojiambo. 2019. Biocontrol strains differentially shift the genetic structure of indigenous soil populations of *Aspergillus flavi*. Frontiers in Microbiology 10:1738.

Liu, X., M. Chen, H. L. Collins, D. W. Onstad, R. T. Roush, Q. Zhang, E. D. Earle, and A. M. Shelton. 2014. Natural enemies delay insect resistance to Bt crops. PLoS ONE 9:e90366.

Martinsen, E. S., S. L. Perkins, and J. J. Schall. 2008. A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. Molecular Phylogenetics and Evolution 47:261–273.

May, G. 2016. Here come the commensals. American Journal of Botany 103:1709–1711.

Messenger, S. L., I. J. Molinieux, and J. J. Bull. 1999. Virulence evolution in a virus obeys a trade-off. Proceedings of the Royal Society B 266:397–404.

Morgan, N. A., and D. B. Sloan. 2015. The hologenome concept: helpful or hollow? PLoS Biology 13:e1002311.

Munkaci, A. B., S. Stoxen, and G. May. 2008. *Ustilago maydis* populations tracked maize through domestication and cultivation in the Americas. Proceedings of the Royal Society B 275:1037–1046.

Nelson, P., and G. May. 2017. Co-evolution between mutualists and parasitoids in symbiotic communities may lead to the evolution of lower virulence. American Naturalist 190:803–817.

_________. 2020. Defensive symbiosis and the evolution of virulence. American Naturalist 196:333–343.

Oliver, K. M., N. A. Moran, and M. S. Hunter. 2005. Variation in resistance to parasitism in aphids is due to symbionts not host genotype. Proceedings of the National Academy of Sciences of the USA 102:12795–12800.

Pan, J. J., A. M. Baumgarten, and G. May. 2008. Effects of host plant environment and *Ustilago maydis* infection on the fungal endophyte community of maize (*Zea mays*). New Phytologist 178:147–156.

Porras-Alfaro, A., and P. Bayman. 2011. Hidden fungi, emergent properties: endophytes and microbiomes. Annual Review of Phytopathology 49:291–315.

R Development Core Team. 2020. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org.

Rigaud, T., M. J. Perrot-Minnot, and M. J. Brown. 2010. Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. Proceedings of the Royal Society B 277:3693–3702.

Rodriguez-Estrada, A. E., W. Jonkers, H. C. Kistler, and G. May. 2012. Interactions between *Fusarium verticillioides*, *Ustilago maydis*, and *Zea mays*: an endophyte, a pathogen, and their shared plant host. Fungal Genetics and Biology 49:578–587.

Rutrecht, S. T., and M. J. Brown. 2008. The life-history impact and implications of multiple parasites for bumble bee queens. International Journal for Parasitology 38:799–808.

Scheuring, I., and D. W. Yu. 2012. How to assemble a beneficial microbiome in three easy steps. Ecology Letters 15:1300–1307.

Schlatter, D., L. Kinkel, L. Thomashow, D. Weller, and T. Paulitz. 2017. Disease suppressive soils: new insights from the soil microbiome. Phytopathology 107:1284–1297.

Shaw, R. G., C. J. Geyer, S. Wagenius, H. H. Hangelbroek, and J. R. Etterson. 2007. Supporting data analysis for "Unifying life-history analysis for inference of fitness and population growth." Technical report 658. School of Statistics, University of Minnesota, Minneapolis. http://www.stat.umn.edu/geyer/aster.

_________. 2008. Unifying life-history analyses for inference of fitness and population growth. American Naturalist 172:E35–E47.

Simon, V., D. D. Ho, and Q. Abdool Karim. 2006. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. Lancet 368:489–504.

Smith, D. L., B. Lucey, L. A. Waller, J. E. Childs, and L. A. Real. 2002. Predicting the spatial dynamics of rabies epidemics on heterogeneous landscapes. Proceedings of the National Academy of Sciences of the USA 99:3668–3672.

Sommer, M. O., and G. Dantas. 2011. Antibiotics and the resistant microbiome. Current Opinion in Microbiology 14:556–563.

Smith, E. J., K. A. Bates, and K. C. King. 2021. Host microbiota can facilitate pathogen infection. PLoS Pathogens 17:e1009514. https://doi.org/10.1371/journal.ppat.1009514.

Schenk. 2018. Emerging microbial biocontrol strategies for plant pathogens. Plant Science 267:102–111.

Toj, H., K. G. Peay, M. Yamamichi, K. Narisawa, K. Hiruma, K. Naito, S. Fukuda, et al. 2018. Core microbiomes for sustainable agrosystems. Nature Plants 4:247–257.

Torto-Alalibo, T., C. W. Collmer, M. Lindeberg, D. Bird, A. Collmer, and B. M. Tyler. 2009. Common and contrasting themes in host cell-targeted effectors from bacterial, fungal, oomycete and nematode plant symbionts described using the gene ontology. BMC Microbiology 9(suppl.):S3.

Tosh, P. K., and L. C. McDonald. 2012. Infection control in the multidrug-resistant era: tending the human microbiome. Clinical Infectious Diseases 54:707–713.

U’Ren, J. M., F. Lutzoni, J. Miadlikowska, N. B. Zimmerman, I. Carbone, G. May, and A. E. Arnold. 2019. Host availability drives distributions of fungal endophytes in the imperiled boreal realm. Nature Ecology and Evolution 3:1430–1437.

van Baalen, M., and M. W. Sabelis. 1995. The dynamics of multiple infection and the evolution of virulence. American Naturalist 146:881–910.

van der Heijden, M. G., and M. Hartmann. 2016. Networking in the plant microbiome. PLoS Biology 14:e1002378.

Van Dyken, J. D., M. J. Muller, K. M. Mack, and M. M. Desai. 2013. Spatial population expansion promotes the evolution of cooperation in an experimental prisoner’s dilemma. Current Biology 23:919–923.
"In December, 1904, I discovered in some water taken by Mr. William G. Lapham from an oozy bank near Afton, Virginia, a large Vampyrella-like specimen [figured], which except for the absence of nuclei and the variable size of the vacuoles answered in detail to Leptophrys elegans." From "Notes on the Genus Leptophrys" by William A. Kepner (The American Naturalist, 1906, 40:335–342).