The Evolution of Symbiosis in Communities

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Dedication

I dedicate this work to my son Kai who is already letting playing with math get in the way of getting real work done.

Abstract

All organisms host a menagerie of symbionts. While harmful pathogens have historically held the attention of researchers, recent technological advances have revealed a cornucopia of benign, and even beneficial, symbionts. Observations that most organisms are party to a wide variety of harmless symbionts are at odds with theory that suggests that infections by multiple symbionts should lead to the evolution of harmful pathogens. Current theory regarding the evolution of symbionts is predicated on the assumption that symbionts receive a reproductive payoff for harming their hosts. Because harming the host, or virulence, indirectly decreases symbiont infection duration, increased symbiont reproduction comes at a cost and leads to a tradeoff. A consequence of this tradeoff is that when multiple symbionts infect the same host the most virulent symbiont receives the highest reproductive payoff while all symbionts suffer decreased infection duration. Consequently, multiple infections are predicted to select for higher virulence, a prediction that runs counter to observation of the plethora of relatively harmless symbionts observed co-infecting most organisms. The three chapters of this thesis seek to bring theory in line with observations of the commonality of co-infecting commensals. The first chapter of this thesis lays out a mathematical model that uses the virulence tradeoff hypothesis to show that multiple infections do not necessarily lead to increased virulence. The second chapter extends the model developed in the first chapter to show that symbiont defense of the host can lead to the evolution of lower virulence. Finally, the third chapter examines genetic variation in virulence and inhibition between symbiont species for fungal symbionts isolated from two populations of maize. Together, this work furthers our understanding of how symbionts evolve in communities and is an important step toward resolving the paradox of ubiquitous benign symbionts.

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Introduction

"No man is an island" has been used for centuries to expresses humans' intimate and universal connection to other humans. It is perhaps fitting that the phrase was coined while its author, John Donne, was stricken with disease (Cox 1973), a state resulting from humans' intimate and universal connection to the invisible microbial world. Just as no man is an island, no organism is an island; every living organism is party to a web of interactions that affects its survival, development, and reproduction (Weiblen 2002; Oliver 2003; Pradeu 2011). Historically, disease causing symbionts have received the most attention from the medical and scientific community. However, recent technological advances have revealed that all organisms, even seemingly healthy ones, are host to a cornucopia of symbionts (Rodriguez et al. 2009; Arumugam et al. 2011). This new world of apparently harmless symbionts poses a problem for the way we traditionally understand the evolution of symbiotic associations. Symbionts must take resources from their hosts to reproduce, therefore we expect some level of harm, or virulence, from most symbionts (Frank 1996). This thesis addresses the mismatch between theory, which predicts that many symbionts will harm their hosts, and observation, which find a cornucopia of symbionts that seem relatively harmless.

The three chapters of this thesis seek to understand the evolutionary dynamics that lead to benign, or even beneficial symbiotic associations. The first two chapters consist of mathematical models for symbiosis evaluated on a continuum between parasitism and

mutualism to determine if multiple infections necessarily cause the evolution of greater virulence. (Bremermann and Pickering 1983; Antia et al. 1994; Nowak and May 1994; van Baalen and Sabelis 1995; Mosquera and Adler 1998; Gandon et al. 2002; de Roode et al. 2005; Caraco et al. 2006; Alizon et al. 2009; Alizon et al. 2013). The third chapter asks if fungal symbionts of maize exhibit genetic variation for traits affecting virulence and competition between symbionts. The evolution of the host-symbiont relationship is closely tied to important questions in evolutionary biology, chiefly the evolution of altruism and the problem of public goods.

The relationship between altruism and symbiont evolution is clear when discussing beneficial symbionts. A symbiont trait that benefits the host at a fitness cost to the symbiont is, by definition, an altruistic trait. The logic behind the study of altruism can also be applied to the study of parasitism. Parasites necessarily exhibit *virulence*, or harm to the host. Virulence is usually understood as part of a tradeoff in which a symbiont receives a reproductive boon from harming its host (Alizon et al. 2009), leading to a tradeoff between transmission rate and infection duration. When other symbionts co-infect the host, host health becomes a public good which can be eroded by symbiont virulence. A symbiont with lower virulence than its competitors harms its host to a lesser degree but does so at a *relative cost to itself*. Therefore the question, "How can symbionts evolve lower virulence?" is equivalent to the question "How can altruism evolve?" The first two chapters of this thesis treat virulence as the effect of symbiont activities on host mortality. Because symbiont morality is tied to host morality for bio-trophic symbionts, the effects of virulence on the host are a public good shared between symbionts.

Virulence can either yield an individual benefit to the symbiont expressing virulence and a public cost to other co-infecting symbionts, or virulence can yield an individual cost and a public good, allowing for the evolution of mutualism and parasitism on a continuum. Together, these chapters ask how communities of relatively benign symbionts can evolve. As a preface for these chapters, in this introduction I discuss the basic mechanisms of natural selection, introduce the problem of public goods and altruism, and finally discuss some solutions to those problems.

Natural Selection and Tradeoffs

Before discussing the role of public goods in symbiotic evolution it may be helpful to review the fundamental forces that shape evolution. Evolution is, in its simplest form, change in allele frequencies over time. In other words, given a genetically based trait *z*, the study of evolution is the study of how trait *z* changes, or Δz. There can be no better illustration of the important aspects of evolution than the Price equation (Price 1970):

$$\Delta z = \mathrm{cov}(z_i, w_i) + E(w_i \Delta z_i) \quad (1)$$

Here change in trait z (Δz) is determined by the covariance between each value of *z* (z_i) and fitness (w_i) and the expected change in the trait value due to fitness (w_i), ($E(w_i \Delta z_i)$). The covariance term captures the effects of natural selection; if the covariance between fitness (w_i) and the trait value (z_i) is positive then the trait value is predicted to increase on average over population i. If the covariance is negative then the trait is deleterious and is predicted to decrease in frequency. An important requirement of natural

selection becomes readily apparent by rewriting the covariance term as a correlation (Frank 1997):

$$\Delta z = \beta \,\mathrm{var}(z) + E(w_i \Delta z_i) \qquad (2)$$

Here β is the correlation coefficient between fitness and trait *z*, or the selection coefficient. In this form we can see that without genetic variation for the trait in the direction of selection, selection cannot affect a change in the trait value even if a trait is under strong selection and β is very large. In other words, evolution requires genetic variation. In this dissertation the first two chapters will focus on the forces that shape selection coefficient, β, on symbiont traits, and the third examines natural populations for evidence of variation in traits affecting organismal interactions. By examining the forces that affect natural selection on symbiotic traits we can shed light on the evolution of benign symbionts.

Natural selection, however, is only half of the Price equation and but one force that affects trait evolution. The second term ($E(w_i \Delta z_i)$) is more nuanced and represents factors other than direct selection that can affect trait evolution. This term can encompass neutral forces such as genetic drift, migration or mutation. Additionally, this term can encompass the effects of multi-level selection, or selection that incorporates both the group and individual level selection, and the public costs and benefits associated with co-infection by other symbionts. The role of public goods and multi-level selection in the evolution of altruism is a key concept in evolutionary biology (Bijma et al. 2007) and will be discussed in greater detail below. In this thesis we modify established models of

parasite virulence evolution (e.g., Lenski and May 1994) to incorporate the effect of public goods and ask how symbionts with low virulence might evolve in a community context.

Equations one and two paint a fairly simplistic picture of natural selection in which a trait will increase without bound as long as genetic variation persists. However, nature is rife with examples of important traits that remain stable over long periods of time despite sufficient variation to allow selection to affect evolution. Examining the Price equation again reveals that, if $\Delta z \approx 0$ while var(z)>0, then β must go to zero. In other words, if a there is variation for a trait but the trait is not changing, then selection on that trait must be constrained in some way. Tradeoffs serve as an important constraint on trait evolution (Asplen et al. 2012). A tradeoff occurs where gain in one aspect of fitness results in loss in fitness associated with another trait. Tradeoffs have been shown to apply to a number of systems as disparate as enzyme kinetics (Savir et al. 2009) to animal foraging behavior (Bonter et al. 2013). A tradeoff between symbiont transmission and infection duration plays a central role in theory regarding the evolution of virulence (Alizon 2009) and is the focus of the first two chapters of this thesis.

When a trait is subject to a tradeoff it is predicted to evolve to an intermediate maximum virulence at equilibrium. The exact value of this state is a function of the form of the tradeoff, the physiology of the organisms, and the particulars of their environment. Tradeoffs often take the form of energy or resource allocation, often represented by the "Y model" in which allocation of a finite resource to one trait comes at the cost of

decreased allocation to other traits (Roff and Fairbairn 2007). For example, growing roots requires a plant to expend resources it could invest in leaves or seeds (Dybzinski et al. 2011). Alternatively, tradeoffs could arise if a behavior yields rewards but also exposes an organism to risk. For example, many animals foraging for food entails exposure to predators (Thaler et al. 2012). In the case of symbionts, drawing too many resources from their host can elicit a host defense response or even kill the host, which also leads to the death of the symbiont (Alizon et al. 2009). In this thesis I explore how the tradeoff between reproduction and infection duration might explain the evolution of benign symbiotic communities.

Public Goods, Altruism, and Parasitism

Tradeoffs might explain why there is a limit on foraging success or the length to which tree roots can grow. In both of these cases, however, the costs and benefits of the tradeoff are borne by the same organism, and this is not the case for host-symbiont interactions. When the costs and benefits of a trait affect different organisms, that trait becomes a public good. Public goods can be beneficial, as when wasps build a nest that may be used by other wasps (Bourke 1999), or detrimental, as when yeasts produce alcohol during fermentation that may limit their own growth and kill off neighboring yeasts (MacLean and Gudelj 2006). When natural selection acts on a trait that is beneficial for the individual but deleterious to the population, decreased population sizes or extirpation can result (Fiegna and Velicer 2003; Kerr et al 2006). Many terms have

been coined to describe the processes in which individuals benefit at the expense of the group, but by far the best known is “The Tragedy of the Commons” (Hardin 1968).

A tragedy of the commons arises whenever the interests of the individual conflict with the interests of the group. Conflict between individual and group interests arise when natural selection favors traits that are deleterious to the population or selects against traits that are advantageous to the population. A clear example of selection against a trait which is advantageous to the population arises with altruistic traits. Altruistic traits are traits that cause an individual to aid others at the expense of itself. For example, some termites have glands which can produce a sticky tar like substance. Termite soldiers will cling to predators and rupture their own abdomens, immobilizing the predator and saving its colony at the cost of their own life (Bordereau et al. 1997). Other examples of altruism are less extreme. For example, many birds work together to raise offspring that are not their own (Grant 1990). In both of these cases individuals incur an individual cost and provide a public benefit.

Conversely, there are also traits that provide an individual benefit at a public cost. A clear example, and the topic of this thesis, arises from co-infection between symbionts (Frank, 1996). When more than one symbiont infects a host and a rapacious symbiont kills the host, all other symbionts die as well. The virulent symbiont receives an individual benefit while inflicting a public cost on other, co-infecting symbionts (Ebert and Mangin 1997). For example, Ebert and Mangin (1997) manipulated the number of co-infections of a microsporidian parasite *Glugoides intestinalis* of *Daphnia magna* by

changing the host death rate. They found that strains of *G. intestinalis* that experienced lower rates of co-infection evolved to be less damaging and maintain longer lasting infections.

The evolution of altruistic traits, which provide public goods at an individual cost, and rapacious traits, which provide an individual benefit while inflicting public costs, may seem to be entirely different phenomena. However, selection *against* a rapacious trait and selection *for* an altruistic trait are mathematically equivalent, as subtracting a negative is equivalent to adding a positive. Therefore, the discussion of the evolution of altruism below applies equally to the evolution of harmful, rapacious traits.

A common tactic for understanding the evolution of altruistic traits is to examine the outcomes of pairwise interactions between just two individuals. The most common metaphor used in pairwise analyses, and the metaphor most closely associated with the study of altruism, is that of the Prisoner's dilemma (Nowak and May 1992). The Prisoner's dilemma models interactions between individuals with two strategies, *cooperation* and *defection*. In this scenario, the greatest global fitness is achieved when two cooperators interact. In contrast, the globally worse outcome arises when two defectors interact, as each receives a low fitness payoff. However, when a cooperator and a defector interact, the defector achieves greater fitness than it would interacting with another defector, and the cooperator achieves lower fitness than between two defectors. Because cooperation results in the greatest global fitness, and cooperators each have lower individual fitness than they would as defectors, cooperation is an altruistic trait.

Under the conditions where a defector always gains from interaction with a cooperator, cooperation can never evolve. In this scenario, an individual will have one of two possible partners, a cooperator or a defector. If the partner is a cooperator, the best immediate individual strategy is to be a defector. If the partner is a defector, the best strategy is to defect and not lose as much as a cooperator. Thus, defectors win in all circumstances and cooperation cannot evolve. Because the best outcomes for the population arise from cooperation, which is costly to the individual, the Prisoner's dilemma results in a tragedy of the commons. This simplistic system overlooks a number of factors that might change interaction outcomes; the model assumes no population structure, the costs and benefits are static, and, by design, is limited to pairwise interactions. In this thesis I develop a model that goes beyond pairwise interactions to ask how interactions between symbionts on a community level might affect the evolution of virulence.

The evolution of altruism can be studied under more general conditions by amending the Price equation to include multi-level selection (Frank 1997). The Price equation given above examines how the trait value of individuals (z_i), denoted by the subscript $\underline{i}$, relates to the fitness of that individual (w_i). Altruistic traits, however, are more complex because they affect fitness on two levels, the individual and the group. The first step in adapting the Price equation is therefore to change the subscript on each term to reflect the average trait value (z_g), and corresponding mean fitness (w_g) of each groups (g), as opposed to each individual:

$$\Delta z = \mathrm{cov}(z_g, w_g) + E(w_g \Delta z_g) \quad (3)$$

As altruistic traits, by definition, always have high group level fitness, one could conclude that the covariance is positive and altruism should evolve easily. However this formulation entirely neglects within group selection, which precisely is where the costs of altruism lay. To remedy this oversight, one can use the original formulation for individual based evolution (Equation 1) to expand the term Δz_g:

$$\Delta z = \mathrm{cov}(z_g, w_g) + E(w_g[\mathrm{cov}(z_{gi}, w_{gi}) + E(w_{gi}\Delta z_{gi})]) \quad (4)$$

Here z_{gi} and w_{gi} are the trait and fitness values of individual i in group g. This equation can be simplified by substituting correlations for the covariances, as in Equation 2, and ignoring the final term ($E(w_{gi}\Delta z_{gi})$). Using β_g for the group level selection coefficient and β_i for the individual level section coefficient, yields:

$$\Delta z = \beta_g \,\mathrm{var}(z_g) + E\,(w_g \beta_g \,\mathrm{var}(z_{gi})) \quad (5)$$

Because altruistic traits confer positive population level fitness and negative individual fitness, β_g must be positive and β_i must be negative. Therefore, Equation 5 shows that the evolution of altruistic traits requires high variance between groups ($\mathrm{var}(z_g)$) and low variance within groups ($\mathrm{var}(z_{gi})$). Returning to the Prisoner's dilemma, individual defectors can outcompete cooperators, but groups of cooperators may out compete groups of defectors. However, just one defector in a population of cooperators will eventually drive those cooperators to extinction. Therefore, for cooperation to be

maintained on a long term basis the variation within populations must be effectively zero, which is a highly unlikely scenario.

Mechanisms for the Evolution of Altruism

How, then, can altruism evolve if within group variation must be unrealistically low? The answer to this question comes in myriad forms and is often system specific, but any solution must either minimize the variance for an altruistic trait within groups ($\mathrm{var}(z_{gi})$) or minimize the costs of an altruistic trait to cooperators. Kin selection is an important mechanism for minimizing within group variance. The second option, changing the costs and benefits of a public good, forms the topic of the first two chapters of this thesis. Therefore, I will briefly introduce kin selection and then discuss the first two chapters of this thesis in which I examine what can happen when the costs and benefits of public goods change with population density.

Kin selection, also known as inclusive fitness, is a method that includes the fitness of relatives when determining the fitness of an individual. For example, an individual whose sister has three offspring would have a higher inclusive fitness than an individual whose sister has zero offspring, all else being equal. Therefore, inclusive fitness captures the benefits that an altruistic trait has on close relatives (Hamilton 1963). Kin selection focuses on the relatedness, r, between individuals, the benefits conveyed to the relative by an altruistic trait, b, and the cost of the altruist of the trait c. Using these definitions, an altruistic trait will be advantageous if, as Hamilton (1963) showed:

$$rb > c \tag{6}$$

Whereas group selection in Equation 5 requires that a population be divided into a number of discrete groups, relatedness (*r* in Equation 6) provides a continuous measurement that can be applied to all members of a population. Kin selection has been incredibly successful in explaining the evolution of many altruistic traits, especially the evolution of social insects (Queller and Strassmann 1992), however its application is not quite as straightforward as the name suggests. Relatedness (*r*), incorporates not only the manner in which two individuals are related to each other, but also the degree to which they are related to the population as a whole (smith et al. 2010). In other words, the relatedness of two brothers in a population of cousins is lower than their relatedness amid a population of strangers. Therefore, kin selection is another way of restating that the evolution of altruism requires low variance within groups and higher variance between groups, albeit with a far more useful definition of "groups".

Altruistic traits can evolve much more readily when organisms can change their strategies based on their partner's actions. In 1981 researchers Robert Axelrod and W. D. Hamilton, author of the seminal papers on kin selection cited above, invited the public to submit strategies for playing the iterated Prisoner's dilemma. These strategies were pitted against each other in series of computer simulations to determine the most competitive strategy. Surprisingly, the winning strategy was the simplistic tit-for-tat, which simply cooperates at first and subsequently copies its partner's behavior (Axelrod and Hamilton 1981). By changing its behavior to match its partner, an organism exhibiting tit-for-tat behavior avoids being taken advantage of by defectors while maintaining high levels of cooperation with other tit-for-taters. In terms of Equation 5, the variance in strategies

among partners is almost zero, facilitating the evolution of cooperation. Tit-for-tat like strategies have been observed in a number of biological systems, where they are often called partner sanctioning, from interactions between fish (Milinski 1987) to exchanges between plants and nitrogen fixing bacteria (Kiers et al. 2006).

Altruistic traits are interesting because they are relatively common (van Dyken and Wade 2012) but, at first glance, seem unlikely to evolve. Group selection, kin selection, and partner sanctioning are all mechanisms that facilitate the evolution of altruism under static costs and benefits. Previous studies have shown that when the benefits of traits change with population density, natural selection can temper or ameliorate evolution of rapacious behavior under a tragedy of the commons (Rankin 2007). In Chapters 1 and 2 of this thesis, I examine the evolution of virulence, a trait involving public goods, when the costs and benefits are variable.

Chapter 1: The evolution of virulence in a symbiotic community

In Chapter 1, I use the classic virulence tradeoff between transmission rate and infection duration but alter it in three ways to ask whether multiple infections necessarily lead to the evolution of more damaging parasites. First, I model the evolution of parasites and mutualists on a continuum. A symbiont's effect on its host is modeled as either positive or negative virulence, and its evolution depends on the biotic context. Second, I place the tradeoff in an ecological context. When infection frequency is low, new infections are easy to establish and selection favors virulent and rapidly reproducing symbionts. When infection frequency is high, new infections are difficult to establish and

selection favors strains with low virulence that can maintain long lasting infections (Lenski and May 1994). Third, I place the tradeoff in a community context by making virulence a public good. Symbionts that aid their hosts act as cooperators, prolonging the infection duration of co-infecting symbionts. Conversely, virulent pathogens act as defectors, killing the host and decreasing their own infection duration of and that of co-infecting symbionts. Using this framework I show that mutualisms can be maintained in the face of multiple infections and that multiple infections do not necessarily lead to the evolution of greater virulence.

Chapter 2: The evolution of virulence and defense of the host

In Chapter 2, I expand on the framework established in Chapter 1 by examining the consequences of symbiont-mediated defense of the host on the evolution of virulence. Interactions between symbionts within a host can be important for the evolution of virulence, especially when symbionts can kill or inhibit other, co-infecting symbionts (Rigaud et al. 2010). Many symbionts may defend their hosts from attack. For example, the endosymbiont of fruit flies, *Wolbachia*, can serve as a defensive symbiont by protecting its host from a virus (Teixeira et al. 2008). Also, fungal endopytes in the genus *Xylaria* can protect the leaves of trees from antagonistic fungal pathogens (Fukasawa et al. 2009). In a macrobiotic example, *Megalomyrmex* ants serve as symbionts of fungus farming ants by protecting them from invasion by more aggressive species of ants (Adams et al. 2012). By defending hosts from their enemies, symbionts can avoid the public costs associated with virulent, co-infecting symbionts. Therefore, I incorporate

symbiont defense of the host into the framework established in chapter 1 to show that symbiont defense of the host can temper the evolution of virulence and preserve mutualisms.

Chapter 3: Natural variation in virulence in a pathogen

Chapters 1 and 2 consist of mathematical models that assume sufficient genetic variation for the evolution of important traits such as virulence and defense of the host. In the third chapter I utilize fungal strains isolated from two different populations of host plants to look for variation in traits important to the host-symbiont relationship and interactions between symbionts. I examine two traits, virulence toward the host in a pathogen, and inhibition of pathogen growth by a defensive symbiont. First, I examine evidence for genetic variation in virulence of the pathogen of maize, *Ustilago maydis*. Second, I examine evidence for genetic variation in the ability of the the fungal endophyte *Fusarium verticillioides*, to inhibit *U. maydis* growth (Rodriguez Estrada et al. 2011). Additionally, to test for factors that may constrain the evolution of virulence in *U. maydis,* I look for a correlation between *U. maydis* growth *in vitro* and virulence toward the plant. Together, the chapters of this thesis help close the gap between theory, which predicts the evolution of communities of virulent pathogens (Frank 1996), and observations that most symbionts are relatively benign (Rodriguez et al. 2009; Arumugam et al. 2011).

Chapter 1: Coevolution between mutualists and parasites in symbiotic communities may lead to the evolution of lower virulence.

ABSTRACT

Many eukaryotes simultaneously harbor a diverse community of parasitic, mutualistic, and commensal microbial symbionts. Although the diversity of these microbial symbiotic communities has recently drawn considerable attention, theory regarding the evolution of interactions among symbionts and with the host is still in nascent stages. Here we evaluate the role of interactions among co-occurring symbionts on the evolution of virulence towards the host. We place the virulence-transmission tradeoff into a community context and model the evolution of symbiont trophic modes along the continuum from parasitism (virulence) to mutualism (negative virulence). To establish a framework for studying multiple infections of the same species, we develop a concept of shared costs, for which the negative consequences of virulence toward the host are shared to varying degrees among species symbiontspecies. We then extend the model to co-infection by multiple species, a parasite and a mutualist. The results shows that mutualism is maintained when shared costs are sufficiently low, while greater virulence and parasitism toward the host are more likely when shared costs are high. Lastly, we show that the presence of a mutualist can ameliorate some costs of pathogen virulence, and consequently, both pathogen and mutualist species evolve to a less virulent state.

Introduction:

Both plant and animal eukaryotes harbor a diverse and abundant microbiome of symbiont species living within their tissues (Arnold et al. 2000; Qin et al. 2010). While the complexity of these symbiotic communities is increasingly recognized (e.g., Piroth et al. 1998; Arnold et al. 2003; Oliver et al. 2003; Márquez et al. 2007), much less is understood about the function of these symbionts (The Human Microbiome Project Consortium 2012; Talbot et al. 2014). However, it is becoming increasing clear that few of these microbes cause disease (Dethlefsen et al. 2007). Consequently, the ecological factors and evolutionary processes that lead to parasitism, mutualism, or commensalism of microbes with their hosts remain an open question (Thrall et al. 2006; Rigaud et al. 2010; Lively et al. 2014). In this work, we address the effects of interactions among co-occurring symbionts on the evolution of virulence.

Parasitism and mutualism describe extremes of a continuous spectrum of symbiont relationships with the host (Johnson et al. 1997; Denison and Kiers 2004), and transitions in symbiotic trophic modes may often occur (Arnold et al. 2009). However, the bodies of theory addressing the evolution of mutualistic and parasitic symbiotic modes have largely developed separately and in parallel. Studies of parasitism typically use deterministic models and explain the evolution of virulence by focusing on the dynamics of host populations, symbiont clearance, and transmission rates (Anderson and May 1979; Ewald 1980). On the other hand, studies of mutualism have most often used models based on game theory (Axelrod and Hamilton 1981) and focus on the conditions required for the maintenance of cooperation against "cheaters". Therefore, the study of

mutualism has focused on mechanisms such as repeated interactions (Doebeli and Knowlton 1998; Doebeli et al. 2004), host sanctions (West et al. 2002), kin selection (Bijma and Aanen 2010; Smith et al. 2010), and mode of transmission (Genkai-Kato and Yamamura 1999). Consequently, we lack an understanding of causes for evolutionary transitions between mutualism and parasitism, especially in the context of the diverse symbiotic communities found in most eukaryotic hosts.

To date, most studies have focused on pairwise host-symbiont interactions (Stanton 2003) and invoke tradeoff models for understanding the constraints on virulence (Asplen et al. 2012). For parasite-host interactions, a negative correlation between the rate and duration of symbiont reproduction should limit the evolution of virulence, here defined as damage to the host that decreases host fitness (Kermack and McKendrick 1932; Anderson and May 1979; May and Anderson 1983; Lenski and May 1994; Gandon et al. 2001; Alizon et al. 2009). While the virulence-transmission tradeoff has been demonstrated in pathogenic interactions (Edmonds et al. 1975; Ebert and Mangin 1997; Mackinnon and Read 1999; Messenger et al. 1999; Ebert 2003; de Roode et al. 2008), it is documented for fewer mutualistic interactions (e.g., Herre and West 1997; Oono et. al 2011) despite its apparent explanatory power (Asplen et al. 2012). The tradeoff involved in mutualism may be viewed in the same light as parasitism; more beneficial interactions with a host should result in longer infection durations but lower transmission rates (Trivers 1971; West et al. 2002; Kiers et al. 2003). In this work, we go beyond pairwise interactions to bring trade-off models into the more realistic ecological context of multiple diverse symbiont communities co-infecting eukaryotic hosts.

A tradeoff between transmission and infection duration may explain why parasites evolve an intermediate level of harm to their host (Levin and Pimentel 1981; Antia et al. 1994; Lenski and May 1994) and outlines the conditions under which mutualisms are favored (Frank 1996; Neuhauser and Farrgione 2004). However, the virulence tradeoff also predicts that within-host competition among co-infecting symbionts will select for increased virulence and select against mutualisms (Bremermann and Pickering 1983; Antia et al. 1994; Nowak and May 1994; van Baalen and Sabelis 1995; Mosquera and Adler 1998; Gandon et al. 2002; de Roode et al. 2005; Caraco et al. 2006; Alizon et al. 2009; Alizon et al. 2013). Therefore, the reality of rampant multiple infections by diverse and largely non-pathogenic symbionts in virtually all eukaryotic hosts pose a paradox: if co-infection selects for more aggressive parasites, how is so much variation in virulence maintained?

To address this paradox we look to the diverse interactions among members of the microbiome as ecological factors that temper the evolution of increased virulence otherwise expected with infection by multiple symbionts (Fellous and Salvaudon 2009; Jaenike et al. 2010; Fenton et al. 2011; May and Nelson 2014). When multiple symbionts infect a host, virulence from one symbiont has the potential to effect all co-infecting symbionts through effects on the shared host. Therefore, we extended single infection models to incorporate virulence as a public cost. In doing so, we place the virulence-transmission tradeoff in a community context to consider the conditions under which symbiotic interactions with hosts might evolve along the continuum between parasitism

and mutualism, and to show that symbionts with diverse trophic interactions with the host can persist under multiple infections.

Methods

Model description

We begin by explicitly defining virulence as a symbiont trait that increases transmission at the cost of harming the host and thus decreases infection duration. Under our definition of virulence, symbionts with positive virulence harm their hosts and are parasites, while symbionts with negative virulence decrease host mortality and are mutualists. To model the relationship between symbiont transmission and infection duration, we incorporate a function (s), which acts as a switch between reproductive and non-reproductive states of the symbiont in the host. We then accommodate multiple infections of varying symbiotic relationship to the host by incorporating an explicit term for shared costs*,* the costs of virulence toward the host experienced by each symbiont in the community within that host. The model with shared costs, both within and between species, explores the conditions under which mutualisms might evolve, and diverse symbionts persist, within the microbial communities occupying eukaryotic hosts.

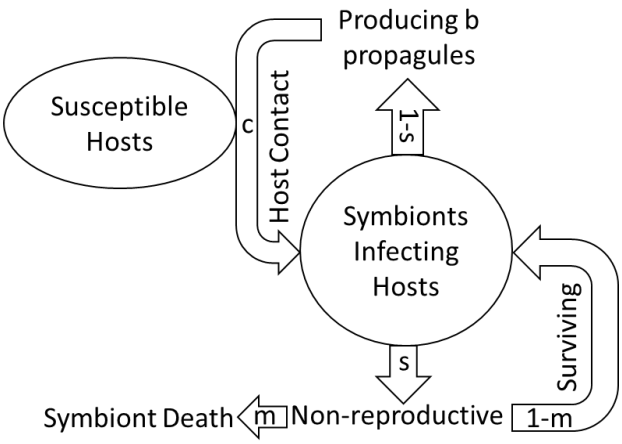

Figure 1.1: Model parameters. Host populations are either susceptible or infected. Establishment of new infections is modeled by the effective contact rate (*c*) and converts susceptible hosts into infected hosts. Symbiont infections have a probability *s* of entering into a non-reproductive state, and in that state, symbionts may die at probabiliy *m* or survive at probability 1-*m* . Infections have a probabilty of 1-*s* of producing *b* propagules. Together, *b* and *c* define the effective transmission rate (*bc*). Virulence manifests as a positive correlation between propagule production (*b*) and the probability of entering a non-reproductive state (s).

Box 1: Terms used.

a_b: Slope of change in propagule production due to virulence

a_s: Slope of change in ***s*** due to virulence

b: Number of propagules produced per infection

b_0: Basal number of propagules produced; value of b when virulence is zero

c: Contact rate with susceptible hosts

I: Number of infections

K: Carrying capacity of hosts

m: Probability of symbiont death while in a non-reproductive state

N: Number of hosts

p: Shared costs of virulence; the fraction of s due to the effects of co-infecting symbionts

r_H: Reproductive rate of uninfected hosts

s: Probability of symbiont entering a non-reproductive state

s_0: Basal probability of entering a non-reproductive state; value of s when virulence is zero

v: Virulence, or increased host mortality due to infection

As in Lenski and May (1994), the host population does not evolve and we assume that the host population size is regulated by a negative density dependent, logistic

function. The host mortality rate is proportional to the mean symbiont virulence weighted by the infection frequency. These assumptions, while simplistic, allow us to use the host population as a feedback on symbiont evolution chiefly through the infection rate. The actual host population size has minimal effect on the results for symbiont evolution. The following equation for host population dynamics results:

$$\frac{dN}{dt} = N\left(\left(1 - \frac{1}{L}\sum_i^L \frac{I_i}{N}\overline{v_i}\right) r_H \left(1 - \frac{N}{K}\right) - \frac{1}{L}\sum_i^L \frac{I_i}{N}\overline{v_i}\right) \quad (1)$$

Here N is the total number of hosts, I_i is the number of hosts infected by symbiont species i, r_H is the growth rate of the host population in absence of any symbionts, L is the number of symbiotic species, and $\overline{v_i}$ is the average virulence of genotypes within species i, and K is the host carrying capacity. The weighted average virulence of symbionts $\left(\frac{1}{L}\sum_i^L \frac{I_i}{N}\overline{v_i}\right)$ represents symbiont induced host mortality and decreases both host population growth rate as well as host population size at equilibrium. By incorporating a switch between reproductive and non-reproductive states which occurs at probability s (Figure 1.1), the rate of change in the number of infections then becomes:

$$\frac{dI}{dt} = I\big((1-s)bc - sm\big) \quad (2)$$

If switched to a non-reproductive state, the symbiont suffers mortality within the host at rate m. Symbionts in a reproductive state $(1-s)$ produce b propagules, which infect a susceptible host upon contact. The contact rate (c) encompasses both transmission to a new host and the availability of susceptible hosts. A feature of this model is that $(1-c)$ describes the probability of death during transmission, which is

subject to ecological context, as compared to *m*, which describes symbiont mortality within the host (Box 1). As *s* appears in both the reproduction term, $(1-s)bc$, and the mortality term (sm), any increase in *s* will result in both an increase in symbiont death and a decrease in transmission. Appropriate to the goal of understanding the effects of interactions among multiple symbionts on the evolution of virulence, *b*, *m*, and *s*, reflect processes within the host, but *c* does not.

Rearranging terms of Equation 2 to express the more familiar net reproductive rate (R_0) yields $R_0 = \frac{(1-s)bc}{sm}$. Note that this formulation differs from the classical form (Anderson and May 1982) in that we focus on symbiont processes. Here, 1-*s* in the numerator represents pathogens in the host that are reproducing with an effective transmission rate, *bc*, to a new host. In the denominator, *sm* represents pathogens that die without reproducing and thus is analogous to host recovery or clearance of the pathogen.

In this work, we use the per capita rate of change in infections $\left(\frac{dI}{Idt}\right)$ of each genotype (*i*), as a measure of fitness, w_i. To model selection pressure on virulence, we examine the relationship between virulence and fitness, of any individual genotype:

$$w_i = (1-s_i)b_i c - s_i m_i \tag{3.1}$$

$$\frac{\delta}{\delta v} w_i = \left((1-s_i)b_i' - s_i' b_i\right)c - s_i' m_i - s_i m_i' \tag{3.2}$$

Here b_i', s_i', m_i' are the derivatives of each function with respect to virulence. Whereas Equation 2 gives the change in total infections for a symbiotic species, Equation 3.1 gives the change in the numbers of an individual strain. Optimal virulence occurs

where Equation 3.2 is zero and reflects an evolutionary stable state (ESS) which is refractory to invasion by genotypes that exhibit either greater or lesser virulence. In this work, we are interested in evolutionary or co-evolutionary outcomes, therefore we focus on the predicted optimal virulence level after sufficient time for natural selection to operate and the system has reached a stable state. Hence, we look for solutions to Equations 2 and 3.2 that yield evolutionary stable states (ESS) at ecological equilibria.

Combining Equation 2, which gives ecological equilibria for an ESS, with Equation 3.2, which gives ESS for each ecological equilibria, produces a system of ecological and evolutionary feedbacks that determine the virulence level at equilibrium. When a new symbiont enters an uninfected, susceptible host population, *c* is large and selection for transmission is predicted to lead to the evolution of more virulent symbiont populations. As the symbiont spreads and susceptible hosts become scarcer, selection pressure for rapid transmission decreases and symbiont virulence evolves to an intermediate level (for a detailed description see Lenski and May 1994). Note that throughout this work we assume sufficient genetic variation and a stable direction of selection over time to generate the predicted effects. The evolution of virulence is constrained by the boundary conditions that *b* is positive and *s, m, c* are all between zero and one. Discontinuities can occur when $\frac{\delta}{\delta v} w_i = 0$ occurs outside of the boundaries conditions ($b > 0$; $I > 0$; $0 \leq s \leq 1$).

Virulence tradeoff.

We incorporate the tradeoff between transmission and duration of infection by allowing symbiont propagule production (*b*), the switch to a non-reproductive states (*s*),

and mortality (m) to increase with virulence (v): $\frac{\delta s}{\delta v}, \frac{\delta b}{\delta v}, \frac{\delta m}{\delta v} > 0$. Additionally, we assume that the contact rate (c) is negatively correlated with infection frequency: $\frac{\delta c}{\delta (I/N)} < 0$ as in Anderson and May (1979). Under these conditions virulence (v) evolves according to Equation 3.2, while the symbiont population changes according to Equation 2. As long as all functions are monotonic, the "competitive exclusion principle" holds and each level of infection frequency (I/N) will yield a single, optimal virulence (Bremermann and Thieme 1989). If infection frequency in low, c is large, and natural selection favors symbionts with high reproductive rates and high virulence. As infection frequency increases, c decreases, and infections that are longer lasting but more slowly reproducing are favored (Lenski and May 1994). Equations 2 and 3 reach equilibrium where $\frac{dI}{dt} = 0$ and $\frac{\delta}{\delta v} w_i = 0$ or:

$$\frac{b'}{b} - \frac{s'}{s(1-s)} - \frac{m'}{m} = 0 \tag{4}$$

Explicit incorporation of multiple infections and shared costs.

The model focuses on the indirect selection on virulence caused by the effects of co-infecting symbionts on host mortality. To do so, we incorporate a term for shared costs; each symbiont gains an individual reward from harvesting host resources, but all co-infecting strains share the costs of effects on host mortality rates, either positive effects of lowering host mortality (mutualism) or negative effects (parasitism). Models incorporating shared costs under multiple infections generally assume a set number of infecting strains, most often two, and that co-infecting strains share costs of virulence

completely (Bremermann and Pickering 1983; van Baalen and Sabelis 1995; Mosquera and Adler 1998; Friesen and Mathias 2010). However, a number of mechanisms such as spatial structure (Lipsitch et al. 1995; Caraco et al. 2006; Kerr et al. 2006), kin structure (Frank 1992), and host sanctions or defense (Antia et al. 1994; Kiers et al. 2003) may prevent the costs of virulence from being shared completely.

The cost of virulence to an individual strain is modeled through *s,* the switch to a non-reproductive state of the parasite, which might result from biological factors such as host resistance or death of the host. Therefore, *s* in Equations 2 and 3 depends on the virulence of an individual strain (v) and the average virulence of all co-infecting strains $(\bar{v})$ (Frank, 1997). Additionally, because the cost of virulence of co-infecting strains will depend on the frequency of the symbiont in the host, we modify $\bar{v}$ by a function, $P\left(\frac{I_x}{N}\right)$, which accounts for infection frequency of that strain and is defined by the overall frequency of multiple infections and the degree to which costs are shared within a host. The shape of $P\left(\frac{I_x}{N}\right)$ is system specific and depends on the probability of reinfection and how virulence manifests within the host. For example, symbionts that grow systemically through the host might generate greater shared costs than do symbionts that grow only in the local region at the point of infection.

While $P\left(\frac{I_x}{N}\right)$ can take on different shapes to encompass different modes of host symbiont interaction, the contact rate (c) must be limited by the infection frequency for the system to reach ecological equilibrium. Thus, while each host can harbor multiple

symbionts, we retain the negative density dependent feedback $\left(\frac{\delta}{\delta\frac{I_x}{N}}c < 0\right)$ required for Equation 2 to reach equilibrium. Assuming that the effects of virulence among multiple infections are additive (as in Bremermann and Pickering 1983), and taking the average over all L species gives the total public cost of virulence: $\frac{1}{L}\sum_i^L P\left(\frac{I_i}{N}\right)\overline{v_i}$ (for a non-additive treatment, see Alizon et al. 2009). The public cost of virulence as used here accounts for the average virulence of each species, the infection frequency, and the degree to which costs are shared among symbiont genotypes and between symbiont species. Finally, we insert linear functions into b, c, s, and m to obtain a set of equations which model the evolution of virulence for symbiotic species x:

$$b_x = a_{bx}v_x + b_{0x} \tag{5.1}$$

$$s_x = a_{sx}v_x + \frac{1}{L}\sum_i^L P\left(\frac{I_i}{N}\right)\overline{v_i} + s_{0x} \tag{5.2}$$

$$m_x = M_x \; is \; constant \; and \;\; 0 < M_x \leq 1 \tag{5.3}$$

$$c_x = 1 - \frac{I_x}{N} \tag{5.4}$$

The terms a_b and a_s give the slopes of the payoff and penalty for virulence, respectively. Greater values of a_b yield more propagules per damage done to the host, while greater values of a_s increase the likelihood that an infection will enter a non-reproductive state due to harming its host. Two parameters expresses symbiont processes when virulence is zero: b_0 is the basal reproduction rate and s_0 is the basal probability of entering a non-reproductive state. For simplicity, we assume m to be a constant; as this

will cause *m* to drop out of Equation 4, the exact value of *M* will have no effect on virulence at equilibrium. Note that *c* as defined by Equation 5.4 will produce a logistic relationship between change in infections and infection frequency. We employ a logistic, density-dependent function to illustrate our results because it is mathematically tractable and commonly used. Many mechanisms can result in density-dependent infection success, such as host immune response (e.g. Portugal et al. 2011), host behavior changes, or direct action of the symbiont (e.g., Folimonova 2012). Note that because the probability of infection for each symbiont species depends only on the infection frequency of that species, symbionts of the same species affect each other both by competing directly for infection sites and through shared costs of virulence whereas symbionts of different species affect each other only indirectly through the shared costs of virulence (Equation 5.2).

For every value of *c* and $\frac{1}{L}\sum_{i}^{L} P\left(\frac{I_i}{N}\right)\overline{v_i}$ there exists an optimal virulence v_x^*, for each species such that $\frac{\delta}{\delta v_x} w_x = 0$. If all strains within a species *x* have virulence v_x^* and the corresponding fitness w_x^*, and a new strain with virulence $\dot{v}_x$ and fitness $\dot{w}_x$ enters the population, the difference in growth rates between the two strains is: $\dot{w}_x - w_x^* = -a_{sx}a_{bx}(v_x^* - \dot{v}_x)^2$. Because a_{sx} and a_{bx} are always positive, $\dot{w}_i$ is always less than w_x^* making v_x^* an evolutionary stable state. This system reaches equilibrium where $\frac{dI_x}{dt} = 0; \frac{\delta}{\delta v_x} w_i = 0$; $\overline{v_x} = v_x^*$ for all *L* species or:

$$v_x^* = \frac{-\left(\frac{1}{L}\sum_i^L P\left(\frac{I_i}{N}\right)\overline{v_i} + s_{0x}\right) \pm \sqrt{\left(\frac{1}{L}\sum_i^L P\left(\frac{I_i}{N}\right)\overline{v_i} + s_{0x}\right) - \frac{a_{sx}b_{0x}}{a_{bx}}}}{a_{sx}} \quad (6)$$

Finding solutions.

The equations presented here yield solutions for any set of parameters given positive tradeoff slopes ($a_{bx} > 0$ and $a_{sx} > 0$) and basal probability of non-reproduction (s_{0x}) between 0 and 1. Solutions can be defined explicitly in the special case of single infections (*P*=0). Under single infections the shared costs are zero $\left(\frac{1}{L}\sum_i^L P\left(\frac{I_i}{N}\right)\overline{v_i} = 0\right)$ and Equation 6 yields explicit solutions which are independent of host dynamics (Equation 1) and host contact dynamics (*c*). Multiple infections (*P*>0), on the other hand, produce non-linear equations in which cannot be solved explicitly. However, numerical solutions can be found by testing values of shared costs $\left(\frac{1}{L}\sum_i^L P\left(\frac{I_i}{N}\right)\overline{v_i}\right)$ for equilibria using Equation 2 and Equation 6. First, a test value of $\frac{1}{L}\sum_i^L P\left(\frac{I_i}{N}\right)\overline{v_i}$ is chosen. This test value of shared costs yields a calculated v_x^* for each species according to Equation 6. Inserting the calculated v_x^* and $\frac{1}{L}\sum_i^L P\left(\frac{I_i}{N}\right)\overline{v_i}$ into Equation 2 yields a calculated $\frac{I_x}{N}$ for each species. Finally, the calculated shared costs using the calculated v_x^* and $\frac{I_x}{N}$ can be compared to the original, test shared costs. Equilibrium occurs where the calculated and test shared costs match. Given the equilibrium values for infection frequency and virulence, Equation 1 then yields the equilibrium host population size. Using a script written in Perl we found equilibria by exhaustively evaluating possible values of total shared costs to an accuracy of 10^{-5}. Stability of each equilibria was then determined by

examining the sign of $\frac{dI_i}{dt}$ and $\frac{\delta}{\delta v} w_i$ at slightly higher and lower virulence and infection frequencies; $\frac{dI_i}{dt}$ and $\frac{\delta}{\delta v} w_i$ is negative both above and below in a stable equilibrium where $\frac{dI_i}{dt}$ and $\frac{\delta}{\delta v} w_i$ are positive to one side of an unstable equilibrium. In cases where the equilibrium falls outside of the boundary conditions ($b > 0$; $I > 0$; $0 \leq s \leq 1$), the symbiont is either determined to go extinct or reach a state of mutualistic non-equilibrium where s is 0.

Results

Two observations can be made regarding Equation 4 without considering the exact shape of the propagule production (b), probability of entering a non-reproductive state (s), or mortality (m) functions. First, because c is not a term in Equation 4, the exact shape of the function describing the probability of contacting a susceptible host has no effect on virulence at equilibrium under single infections. Second, if the three functions *b, m,* or *s*, are not functions of virulence, the values of these parameters have no effect on virulence at equilibrium because the derivative with respect to v is zero.

We focus on three scenarios: single infections, multiple infections of the same species, and co-infection of two species, a potential pathogen and a potential mutualist. In all cases, we assume b and s are linear functions of virulence, M is constant, and c is density-dependent function as shown in Equations 5.1 through 5.4 above.

Single Infections.

In the special case of single infections where $P\left(\frac{I}{N}\right) = 0$, the system can be solved explicitly giving virulence at equilibrium:

$$v^* = \frac{-s_0 \pm \sqrt{s_0 - \frac{a_s b_0}{a_b}}}{a_s} \tag{7}$$

As can be seen in Equation 7, increasing the fecundity payoff for virulence, a_b, leads to greater levels of virulence at equilibrium. Conversely, increasing the penalty for virulence, a_s, as the switch to a non-reproductive state (*s*), leads to lower levels of virulence at equilibrium. A key finding is that a greater basal probability of being in a non-reproductive state, s_0, leads to the evolution of greater virulence. Because we are modeling virulence as the net cost to the host, negative *v* corresponds to a mutualistic symbiont that reduces host mortality, thus prolonging infection duration at the cost of decreasing symbiont transmission. When $b_0 < \frac{a_b}{a_s}(s_0 \pm {s_0}^2)$, v^* is less than zero, and mutualism will evolve. Thus, incorporating symbiont reproduction while in a commensal state with no virulence (b_0) allows for the evolution of mutualisms.

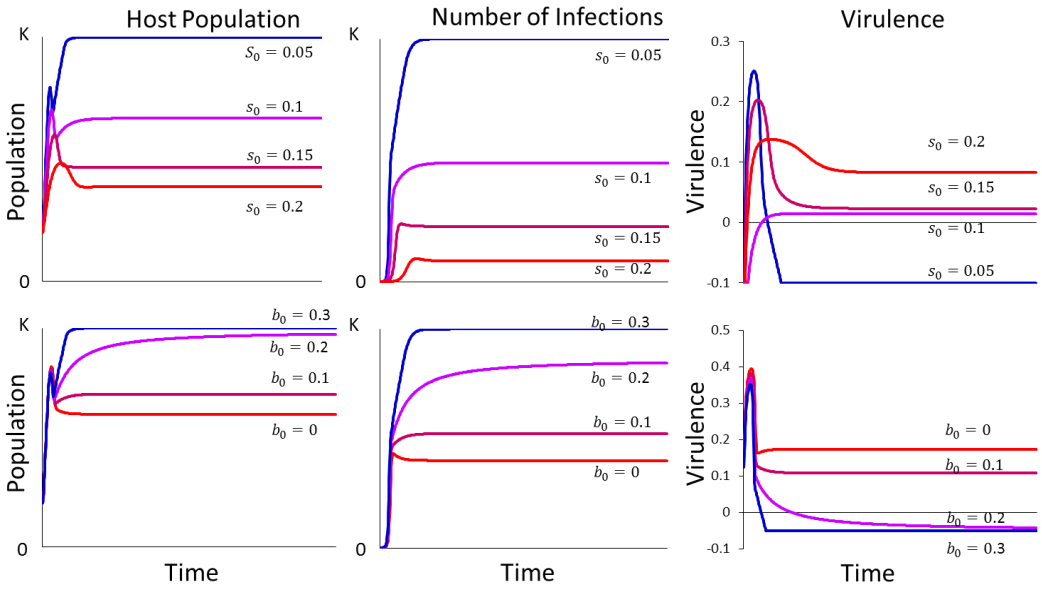

Figure 1.2: Virulence evolution for single infections. The top panel depicts the effects of varying the basal probability of entering a non-reproductive state, s_0, on the host population size N (Equation 1), the number of infections, and equilibrium virulence over time (b_0=0.3; r_H=8; a_s=1; M=1). The bottom panel depicts the effect of varying the basal propagule production rate, b_0, for the host population size, number of infections, and equilibrium virulence over time (s_o=0.05; r_H=8; a_s=1; M=1). Positive virulence indicates parasitism, negative virulence indicates mutualism, and zero virulence indicates commensalism. Mutualisms may evolve when s_o is low and b_0 is relatively high.

Figure 1.2 depicts the results of a discrete time series analysis where the host population size and the symbiont infection frequency begin small relative to *K* and change according to Equations 1 and 2, and where virulence evolves according to

Equation 3. At each time step, *t*, the population reaches local equilibrium where $\frac{dI}{dt} = 0$ for the average virulence during the previous time step, *t*-1. Additionally, average virulence evolves to the evolutionary stable state given the infection frequency at *t*-1. In all cases, immediately upon entering a population of susceptible hosts, high virulence is advantageous and we find a spike of virulence even among potential mutualists. As the symbiont spreads throughout the population, susceptible hosts are harder to find and lower virulence is more advantageous. If b_0 is sufficiently high and s_0 is sufficiently low (s_0 =0.05 and b_0> 0.1 in Figure 1.2), the symbiont evolves negative virulence and acts as a mutualist, and the host population approaches *K*. In cases of positive virulence (parasitism), the host population equilibrates below the carrying capacity. Interestingly, stable commensal states (virulence near 0) may be obtained at intermediate values of b_0 and s_0. However, most ranges of parameter values have a cost to host population size, suggesting selection for host resistance if we had allowed the host population to evolve.

For the evolution of virulence, the two results shown with s_0 = 0.05 (top panel) and b_0 = 0.3 (bottom panel) are special cases where the term under the square root in Equation 7, $s_0 - \frac{a_s b_0}{a_b}$, is negative and equilibrium is never reached. Here optimal virulence occurs where $s < 0$. Because this violates the boundary condition that *s* must be positive, the system enters a non-equilibrium state where virulence evolves as low as possible, $-\frac{s_0}{a_s}$, and the infection frequency approaches unity. By separating transmission affected by the virulence tradeoff, $a_b v$, from the basal transmission rate, b_0, and

incorporating ecological feedbacks of the host population size, these equations allow the full spectrum of mutualistic to parasitic interactions with the host to evolve.

Multiple infections by a single species.

To find solutions for multiple infections by different strains of a single species, we make $P\left(\frac{I_x}{N}\right) = p\frac{I_x}{N}$, where p represents fractional shared costs, or the fraction of virulence that affects other symbionts via effects on mortality of the shared host. Also, we have $a_{sx} = (1-p)$, the fraction of a strain's virulence that affects its own probability of being in a non-reproductive state. Thus, this is a special case where the deleterious effect of a strain's own virulence decreases as the effect of other strains' virulence increases and the total cost of virulence is independent of *p*. The parameter *p* thus changes the manner in which each strain is affected by its own virulence and that of co-infecting strains. Additionally, as the level of shared costs is strongly affected by the infection frequency $\left(\frac{I_x}{N}\right)$, the results now depend upon *c*, the probability of infection defined by Equation 5.4.

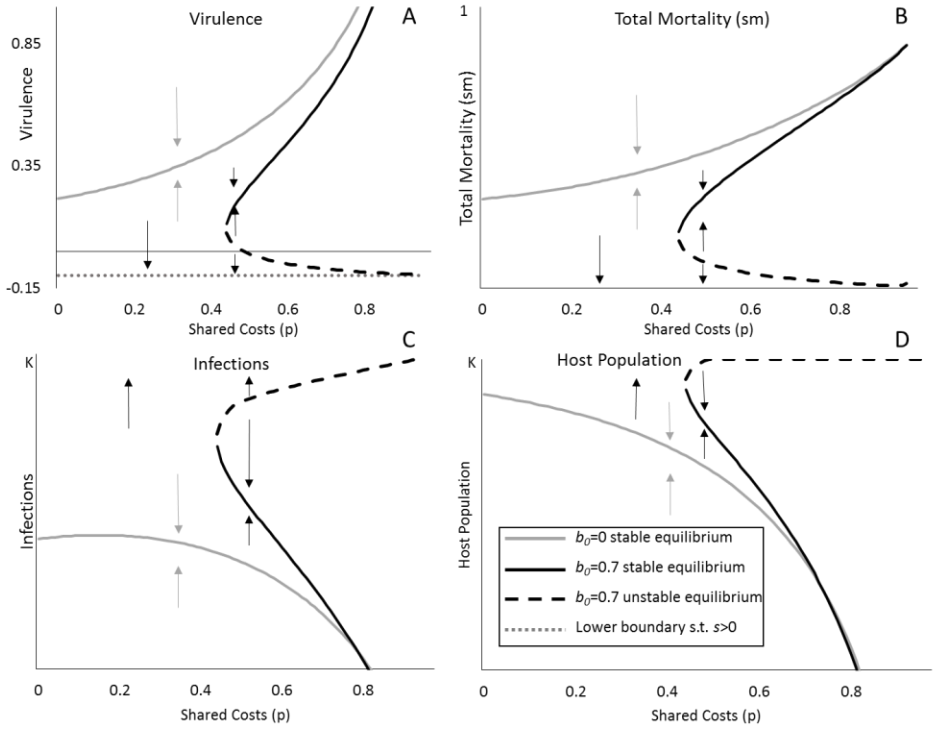

Figure 1.3: The effects of shared costs on the evolutionary stable state for virulence (A), total symbiont mortality (B), number of infections (C), and host population size (D). Grey lines indicate equilibrium states for an obligate parasite (b_0=0), and black lines indicate equilibrium states for a potential mutualist (b_0=0.7). Solid lines indicate stable equilibria, and dashed lines indicate unstable equilibria. Guide arrows illustrate the dynamics of the potential mutualist (black) and the potential pathogen (grey).

The results of the model depend strongly on the degree of cost sharing and the basal production rate of infectious propagules (b_0). If $b_0 = 0$ (shown by the grey lines in Figure 1.3), symbiont reproduction always increases host mortality and the symbiont is

an obligate parasite. Virulence will increase as shared costs increase, pushing the symbiont total mortality rate (sm) towards one (Figure 1.3B), and both the parasite (Figure 1.3C) and the host (Figure 1.3D) go extinct, a tragedy of the commons. However, if the symbiont is a potential mutualist (black lines in Figure 1.3), one of three states may emerge. If cost sharing is sufficiently low, the system moves to a state of mutualistic non-equilibrium. Virulence decreases until s reaches the lower boundary condition at s=0, and infection frequency and host population size both increase to carrying capacity (K). As shared costs increase, stable and unstable states emerge and the system becomes sensitive to initial conditions. If starting from a mutualistic state at values below those of the unstable equilibrium, shown by the dashed black line in Figure 1.3A, symbionts evolve ever decreasing virulence. However, if starting from a less mutualistic state defined by values above the dashed black line in Figure 1.3A, selection favors greater virulence and symbionts evolve to the parasitic stable state. As with the obligate parasite, as shared costs increase virulence increases hyperbolically and ultimately the host and symbiont go extinct as total mortality approaches one.

Figure 1.3 depicts a scenario in which both public and individual costs of virulence increase with multiple infections and result in a classic tragedy of the commons. However, not all biological systems with shared costs experience a tragedy of the commons (Rankin et al. 2007). In the results above, the costs of an individual strain's virulence to itself (a_s) decreases as the cost of co-infecting strains virulence (p) increases. The total cost of virulence among all symbionts remains constant but the costs

are shifted from an individual strain that would receive the reproductive benefit of its own virulence to other co-infecting strains.

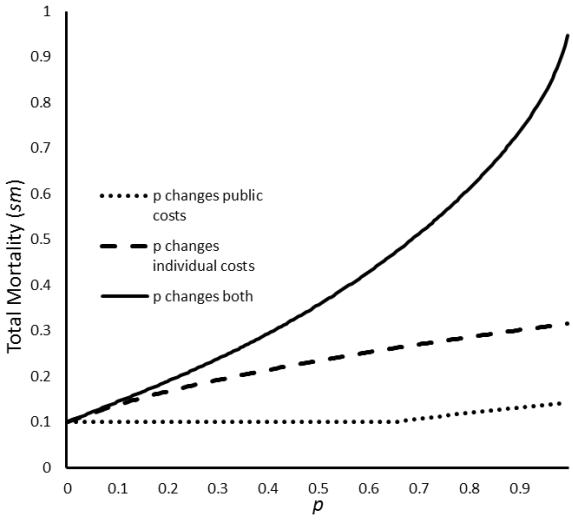

Figure 1.4: Dissecting the effects of individual and public shared costs among multiple infections of the same species. Starting parameter values were chosen that yield a commensal symbiont ($v^*=0$) under single infections ($p=0$, $b_0=0.18$; $a_b=2$; $s_0=0.1$; $M=1$). With these parameter values, total mortality (sm) above 0.1 indicates parasitism, total mortality at 0.1 indicates commensalism. Stable equilibria for sm are shown for the case in which the public cost of virulence changes with shared costs ($s = v + pIv + s_0$; dotted line), where individual costs of virulence change with shared costs ($s = (1-p)v + s_0$; dashed line), and where both individual and public costs change together with shared costs ($s = (1-p)v + pIv + s_0$; solid line). All other parameters remain the same for the three conditions. Shared costs result in a tragedy of the commons (solid line where $sm \rightarrow 1$) only when symbionts

simultaneously experience lower costs of their own virulence and greater public costs from co-infecting strains.

A strength of our approach is that we can decouple the effects of individual and shared costs by changing a_s independently from p. We refer to a_s as the individual cost of virulence and p as the public cost of virulence and choose a parameter set that produces a commensal symbiont under single infections (see Figure 1.3). Because virulence reaches very large values if a_s is small, but total symbiont mortality (sm) stays between zero and one, we illustrate the effect of manipulating public costs independently from individual costs of virulence by examining change in total symbiont mortality (Figure 1.4). If public costs of virulence increase while the individual costs remains constant, the commensal state is maintained until public costs become very high (p>0.65), at which point the symbiont adopts a more parasitic strategy (Figure 1.4, dotted line). Conversely, if the individual costs of virulence decrease and public costs of virulence remain constant, the commensal state is lost immediately and total mortality increases much more quickly than when public costs increases alone (Figure 1.4, dashed line). Furthermore, public and individual costs have non-additive effects on virulence when changed in concert. When public costs increase at the same time as individual costs decrease, virulence increases much faster than the previous two cases. Total mortality approaches one hyperbolically as $p \rightarrow 1$, resulting in a tragedy of the commons (Figure 1.4, solid line).

Co-infection by multiple species.

We are primarily interested in the evolution and maintenance of mutualisms in the face of co-infecting parasites and model this by using starting conditions that yield a mutualist ($\boldsymbol{a_{bm} = 2, b_{0m} = 0.4, s_{0m} = 0.1}$) and a parasite $\boldsymbol{\left(a_{bp} = 4, b_{0p} = 0, s_{0p} = 0.1\right)}$ when shared costs are zero. When evolving alone, each symbiont is affected only by the average virulence of its own species, whereas under coevolution each symbiont is affected by the average virulence of its own as well as the other co-infecting species.

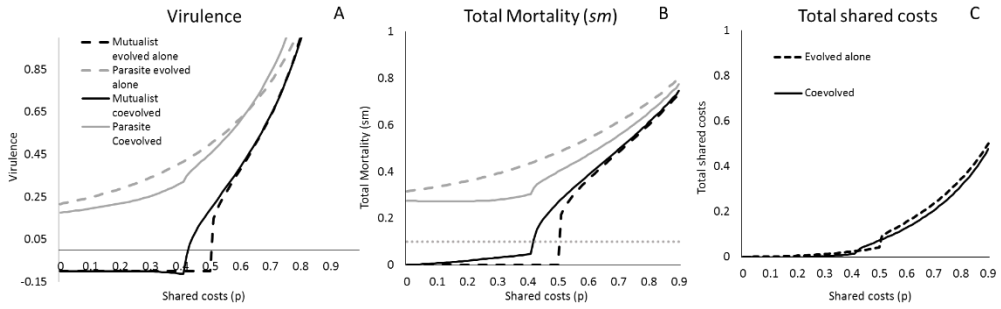

Figure 1.5: Coevolution of a parasite and a mutualist. The evolutionary stable state at ecological equilibrium is shown for virulence (A) total mortality of symbionts (B) and total shared costs $\left(\frac{1}{L}\sum_i^L p\frac{I_i}{N}\bar{v}_i\right)$ among symbionts (C). The status of potential mutualists is shown in black and obligate parasites in grey. To compare results under coevolution and single species evolution, results are shown for the single species, shared cost model (dashed lines). The basal probability of entering a non-reproductive state is indicated by a grey dotted line ($s_0 = 0.1$) in panel B. Symbionts exhibiting virulence below zero and total mortality less than $s_0 = 0.1$ are mutualists, and those exhibiting virulence greater than $s_0 = 0.1$ are parasites.

As shown in Figure 1.5, the level of shared costs is critical; if shared costs are sufficiently low, the presence of a mutualist can mitigate some of the damage done by a pathogen. The decrease in host mortality mediated by the mutualist selects for less virulent pathogens and leads to lower equilibrium levels of virulence for both species than if both species had been evolving alone (Figure 1.5A). As shared costs increase, total mortality for both symbionts sharply increases (Figure 1.5B), the mutualist switches to a parasitic state. As the host is now subject to two parasites, total shared costs are higher than expected in the case of each species was evolving independently (Figure 1.5C). As shared costs increase further, the differences between coevolving and evolving alone disappears as both symbiont's virulence increases hyperbolically and lead to a tragedy of the commons.

Discussion

In this work we develop a model to address the paradox of the many diverse microorganisms that live within most eukaryotic hosts without causing disease. We place virulence on a continuum of positive and negative values to examine the evolution of symbiotic associations with the host in the context of multiple infections. In our model, as in classic models (Bremermann and Pickering 1983, van Baalen and Sabelis 1995, Mosquera 1998, Alizon 2009), co-infecting symbionts share the cost of each other's virulence. We modeled the shared costs of virulence on a quantitative scale to capture the myriad factors that may limit the degree to which costs are shared; spatial structure

(Lipsitch et al. 1995; Orians and Jones 2001; Kerr et al. 2006), kin selection (Frank 1996), host sanctions (Wilkinson and Sherratt 2001; Kiers et al. 2003) and immunity (Antia et al. 1994). We then examined a system in which different symbiont species do not compete directly for infection sites or host resources but do affect each other indirectly through their impact on the shared host. Placing the virulence tradeoff in a community context, we are able to show that mutualisms can be stable under multiple infections and that commensalism is an unexpectedly stable state under a wide range of ecological conditions.

Model setup

The model examines the evolution of symbionts along the continuum of virulence from parasitism to mutualism under co-infection by multiple, diverse species. We use a tradeoff between transmission and the probability of a symbiont entering a non-reproductive and potentially fatal state to model the evolution of symbiont virulence. For generality, we use a linear relationship between the costs and benefits of virulence in lieu of more specific mechanisms related to factors affecting host-symbiont interactions such as host quality (May and Anderson 1983), host sanctions (West et al. 2002), waste product utilization (Genkai-Kato and Yamamura 1999), retaliatory behavior (Wilkinson and Sherratt 2001), or vertical transmission (Foster and Wenseleers 2006). Coupling the probability of entering a non-reproductive state to virulence allows incorporation of the full spectrum of host-symbiont relationships; positive values of virulence represent parasitism, and negative values represent mutualism. Just as virulence exists on a

continuum, the degree to which virulence of one symbiont affects other strains' mortality also manifests on a continuum. Therefore, to model outcomes under multiple infections we introduce a term, *p*, which accounts for the fraction of costs of virulence that are shared among co-infecting symbionts; *p* can vary between zero (single infection) to one (multiple infections where all costs are shared). Using an explicit term of cost sharing enables an examination of coevolution between multiple symbionts that engage in very different relationships with their hosts, such as parasites and mutualists.

Single Infections

Results show that increased virulence evolves under conditions similar to those shown in classic models under single infections where selection for increased transmission causes selection for greater virulence and results in a lower duration of infection (Bremermann and Pickering1983, Gandon et al. (2001). For example, greater host mortality or a greater immune response may lead to a greater probability that the parasite switches to a non-reproductive state and the evolution of increased parasite virulence. Interestingly, under parameter values allowing symbiont reproduction at zero virulence (a commensal state), the tradeoff between transmission and virulence may allow the evolution of mutualism instead of parasitism. Further, in contrast to parasitism, mutualisms emerge from non-equilibrium states as well as from stable equilibrium states. These non-equilibrium mutualistic states are obtained with parameter values under which symbionts receive little benefit for virulence relative to their rate of reproduction as a commensal. Under these parameters, selection favors ever more mutualistic symbionts

with infection frequencies approaching 100%. These mutualistic non-equilibrium states may explain the common observation of mutualists exhibiting tight vertical transmission or in systems with host sanctioning against parasites (e.g. Kikuchi et al. 2007; Ferrari et al. 2011; Melkonian et al. 2013).

Multiple infections

As single infections are the exception rather than the rule, we expanded the model to incorporate multiple infections of the same species. Similar to the models developed in Antia et al. (1994) and Mosquera and Adler (1998), our results show that multiple infections with high levels of shared costs may cause selection for increased virulence and result in a tragedy of the commons. Here we contribute an examination of shared costs among symbionts in a quantitative, continuous framework. The results under low or moderate shared costs demonstrate that mutualisms may evolve and be stably maintained under multiple infections. Moreover, our model shows that a tragedy of the commons due to multiple infections requires increasing shared costs coupled with decreasing penalties for a strain's own virulence. These results suggests that co-infection may not always cause an increased cost of a strain's virulence to itself and help to explain the common observation that runaway virulence is rare (Rigaud et al. 2010). Additionally, we show that commensalism might be particularly robust to the effects of shared costs. While under single infections virulence increases continuously as the basal probability of entering a non-reproductive state increases, with multiple infections, symbionts maintain virulence levels close to zero, and thus are commensals, over a wide range of shared

costs. The stability of commensalism suggests adaptation to the ecological context of multiple infections, rather than the latent stage of pathogens (as in Sorrell et al. 2009) held in check by host tolerance (as in Miller et al. 2006), or more simply, a category in which we place symbionts whose function is otherwise unknown.

An unexpected outcome of incorporating both virulence and shared costs on a continuum of values is the emergence of stable and unstable equilibria. The unstable equilibria suggest that highly mutualistic, but fragile, communities could evolve if populations are founded by sufficiently mutualistic symbionts. As infection by mutualistic genotypes precludes infection by more pathogenic strains of the same species, invasion by a pathogenic strain would be successful only if accompanied by an ecological disturbance resulting in decreased mutualist infection frequencies. While our model predicts competitive exclusion of all but a single strain under direct competition within a species, the models presented in Bronstein et al. (2003) and Morris et al. (2003) have shown that spatial structure can also lead to ecological coexistence between mutualists and parasites. Our result that multiple infection can lead to either mutualistic or parasitic symbionts depending on the initial community composition confirm findings of Bronstein et al. (2003) and Morris et al. (2003) and demonstrate the importance of treating ecological context in models for the evolution of virulence.

The importance of transmission in virulence evolution is illustrated by the role of the function determining the probability of contact with susceptible hosts (c). Under single infections, the contact function affects the evolution of virulence away from equilibrium but has no effect on virulence at equilibrium. Under multiple infections

however, the contact function becomes important as it affects equilibrium infection frequency, which in turn affects virulence. Incorporating infection frequency into shared costs produces an ecological feedback in which higher virulence typically leads to lower infection frequencies, thus tempering the effects of highly virulent strains on total shared costs.

Co-infection by multiple species

We applied the virulence tradeoff to coevolution between a potential mutualist and an obligate pathogen interacting within a eukaryotic host. As in previous work, (Bronstein et al. 2003, Doebeli et al. 2004), our model incorporates the interaction between symbionts as the shared costs of virulence on a common host. Our model improves upon previous efforts to study infection by multiple species by treating both virulence and shared costs on a continuous scale. We are able to show that mutualisms may persist in the face of co-infection by more virulent symbionts. Indeed, we show that when shared costs are moderate, the presence of a mutualist can lead to the evolution of lower levels of virulence in a parasite and therefore lower host mortality. Our unique approach to incorporating the effects of multiple infections by making virulence a public cost and modeling parasitism and mutualism on a continuum captures the full range of outcomes associated with co-infection by pathogens and mutualists.

Future directions

The model presented here is flexible enough to accommodate more complex systems because additional parameters can be incorporated into the additive framework. For example, host evolution could be included allowing examination of multihost symbionts (Woolhouse et al. 2001; Gandon 2004), and the evolution of host resistance (Roy and Kirchner 2000; Dybdahl and Storfer 2003; Restif and Koella 2004) or tolerance (Inglese and Paul 2006). Additionally, my model includes a single symbiont trait, virulence, and symbionts interact with each other only indirectly through the effects of their virulence on a shared host. In reality, symbionts engage in a multifaceted array of direct and indirect interactions with other symbionts and the expression of many traits, such as secondary metabolites, may depend on ecological context and host genotype (Bergstrom et al. 1999; Rooney and Klein 2002; Bronstein et al. 2003; Inglis et al. 2009; Jones et al. 2009; Dyszel et al. 2010; Rodriguez Estrada et al. 2012). Incorporating direct interactions and plasticity will add further depth to the investigation of the effect of community on symbiont virulence.

Conclusion

The results of my model show that understanding the manner in which co-infecting symbionts interact is vital to explaining the diversity of interactions between these symbionts and their hosts. The results suggest that multiple infections do not necessarily lead to the evolution of increased virulence if there are mechanisms that limit the degree to which costs of virulence are shared among symbionts. Moreover, because symbionts within a host share the benefits of mutualism as well as the costs of parasitism, pathogens as well as mutualists will benefit from lower host mortality due to mutualists.

Thus, while co-infection by different parasites often leads to the evolution of more damaging parasites, co-infection by parasites and mutualists can result in lower virulence in the parasite. Together, results show that multiple infections by a diverse community of symbionts may temper the evolution of virulence rather than exacerbate it.

Chapter 2: Defensive symbiosis and the evolution of virulence

ABSTRACT

Chapter 1 of this thesis examines the effect of multiple infections on the evolution of virulence. We modeled mutualism and parasitism on a continuum and introduced the concept of shared costs of virulence. We showed that, while multiple infections can select for increased virulence, if shared costs are low, mutualism can evolve under multiple infections. These results were dependent on the assumption that symbionts only affect each other indirectly through their effects on a shared host. In Chapter 2 of this thesis we build upon the work laid out in Chapter 1 by examining the evolution of virulence in the presence of symbionts that defend their hosts from enemies. Defensive symbionts may protect their hosts from enemies while causing little to no damage themselves and express both low virulence and traits that repel, kill, or inhibit a host's grazers, pathogens, or predators. While environments rife with enemies might cause selection for defensive traits, theory suggests that enemy rich environments also select for greater virulence because the most exploitive pathogens will reap the benefits of harming the host. Thus, co-infection of a defensive symbiont and an enemy of the host is predicted to select for both more virulent pathogens and for greater defensive traits. In this chapter, we build a model that incorporates the evolution of defense and virulence as two independent traits. Symbionts can invest in defense that ameliorates the costs associated with co-infection with deleterious parasites. As in Chapter 1, a symbiont's direct effect on host mortality (virulence) is incorporated as a continuous trait, allowing symbionts to evolve between mutualism and parasitism. The model shows that, while defense can lead to higher

virulence when defensive traits are costly, defense largely leads to the evolution of lower virulence and facilitates mutualism.

Introduction:

Symbiotic organisms are ubiquitous and can have an array of effects on their hosts, from deleterious disease-causing parasitism, to beneficial, mutualistic effects enhancing host survival (Oliver 2005; Roossinck 2011) or reproduction (Bronstein et al. 2003). Most symbiotic interactions take place in the context of an entire community of diverse, symbiotic organisms (Stanton 2003; Rigaud et al. 2010; Thompson et. al 2013) with a single host often party to multiple symbioses. Multiple infections affect symbiont evolution in two ways. First, multiple infections can cause either deleterious (Ebert and Mangin 1997) or beneficial (Rumbaugh et al. 2012) effects that are shared among co-infecting symbionts. Second, when multiple symbionts infect the same host those symbionts can engage in direct interactions and potentially inhibit each other's growth or reproduction. When the costs of infection are shared between symbionts, selection is widely expected to favor increased virulence (Nowak and May 1994; May and Nowak 1995). Conversely, when symbionts inhibit other, more damaging symbionts, selection is expected to favor less virulent symbionts (Fenton et al. 2011; Jones et al. 2011). In this work we ask whether the presence of defensive symbionts cause selection for the evolution and maintenance of low virulence under multiple infections.

Symbionts that defend their host have been observed in many systems (Carroll 1988; Madden and Young 1992; Balmer et al. 2009; Jaenike et al. 2010; Vittecoq et al.

2012). For example, some fungal endophytes in cacao have been shown to protect the host against plant pathogens from the genus *Phytophthora* (Arnold et al. 2003). Studies of aphids have shown that endosymbionts can protect against parasitoid wasps (Oliver et al. 2008). Additionally, the fungal endophyte *Neotyphodium*, has been shown to protect its grass host from herbivores (Clay and Schardl 2002). While defensive symbioses have been documented in a number of systems, we know less about the evolutionary and ecological processes that maintain defensive symbioses (May and Nelson 2014). In this work we focus on defense of the host as a mechanism that protects the host from other symbionts and thereby decreases the shared costs of virulence. We lay out a model that synthesizes two important effects of symbionts on the evolution of virulence: the direct effects of the symbiont on the host, virulence, and the indirect effects of a symbiont killing or inhibiting co-infecting damaging symbionts, defense.

Theory regarding the evolution of virulence is predicated on the assumption of a tradeoff between transmission rate and infection duration (West et al 2002; Alizon et al. 2008). That trade-off can take the form that symbiont transmission requires harming their hosts (Alizon et al. 2008) or that symbionts pay a cost for aiding the host (West et al 2002). Numerous studies have found a tradeoff between symbiont transmission and the degree to which a symbiont harms its host (Messenger et al. 1999, Abedon et al. 2003; Ebert et al. 2004; Sachs and Wilcox 2006; deRoode et al. 2008; Mackinnon et al. 2008). For example, deRoode et al. (2008) found that strains of a protozoan parasite with higher transmission rates also killed their butterfly hosts faster than strains with lower transmission rates. For a castrating parasite of *Daphnia*, Ebert et al. (2004) found that

host and parasite reproduction faced a tradeoff as increasing parasite fecundity was associated with a decrease in host fecundity. In an example involving an algal symbiont of jellyfish, increased transmission of the algae between hosts was associated with decreased host fecundity and survival (Sachs and Wilcox 2006). Furthermore, malaria, an important human pathogen, has been shown to show a positive correlation between transmissibility and virulence in mice (Mackinnon et al. 2008). Additionally, Abedon et al. (2003) found that bacteriophage evolved shorter latency periods, and greater virulence, when bacterial populations were dense. The highly virulent strains were then out competed by less virulent strains when bacterial populations were less dense. Therefore Abedon et al. (2003) show that virulence provides a transmission benefit but at the price of decreased infection duration for the phage, indicating a tradeoff. While other studies have examined the effect of symbionts on host growth and reproduction (Messenger et al. 1999), in this work we focus specifically on a symbionts effect on its host's mortality.

Because biotrophic symbionts require a living host for growth and reproduction, any change in the host's mortality, or the mortality of host tissue, affects the symbionts mortality as well (Bremmerman and Pickering 1983). Therefore, a tradeoff between symbiont transmission rate and host mortality promotes the evolution of stable levels of benefits or harm to the host (Alizon et al. 2008; Barrett et al. 2011). The evolutionarily stable level of virulence towards the host depends on many factors, including the mortality rate of the host, host defenses, and symbiont transmission (Levin and Pimentel 1981; Lenski and May 1994; Williams and Day 2001; Sorrell et al. 2009). While

symbiont mortality is tied to host mortality, changes in host and symbiont mortality need not be symmetric. For example, a pathogen such as the rust fungus *Puccinia eupatorii,* may induce leaf senescence and thus kill its own substrate for growth,but the effects on its herbaceous host might be relatively minor (Goodall et al. 2012). Therefore, we construct our model to reflect evolutionary and ecological forces as they affect each symbiotic species, as opposed to the host.

Hosts rarely interact with a single symbiont exclusively, making multiple infections the rule rather than the exception (Arnold et al. 2003; Saikonnen 2007). When multiple symbionts infect the same host, they share costs and benefits associated with each symbiont.. Such shared costs can manifest in terms of resource competition (Choisy and deRoode 2010) or host mortality (Ebert and Mangin 1997). Multiple infections are predicted to select for more virulent and less beneficial symbionts when each symbiont receives individual benefits from its own virulence (Bremmerman and Pickering 1983; van Baalen and Sabelis 1995; Mosquera and Adler 1998; West et al 2002; Alizon et al. 2008; Friesen and Mathias 2010). That prediction has been borne out empirically (Ebert and Mangin 1997). It is important to note that current models (e.g. Bremmerman and Pickering 1983; van Baalen and Sabelis 1995; Mosquera and Adler 1998; Alizon et al. 2008; Friesen and Mathias 2010) only treat multiple parasites and implicitly assume that the costs of virulence are shared fully between symbionts. Thus, these models do not address the effects of multiple infection on the evolution of virulence in a quantitative fashion, and cannot incorporate the evolution of symbiotic defense of the host. When pathogen virulence itself becomes a shared trait, as with bacteriophage assembly (Turner

and Chao 1999) or siderophore production(Griffin et al. 2004), multiple infections can actually lead to decreased virulence by allowing cheaters that do not contribute to collective virulence (Alizon and Lion 2011; Rumbaugh et al. 2012). In this model we incorporate the shared costs of co-infection on a continuous scale in order to capture the varying degrees of interaction observed between symbionts in nature.

When multiple symbionts interact within a host, defensive symbionts can gain a fitness advantage by inhibiting enemies relative to symbionts that do not defend against enemies, thereby decreasing the public costs associated with virulent coinfectors (Jones et al. 2011). When taken together, the evolution of defense and the evolution of virulence produce an evolutionary conundrum. On one hand, we expect diverse communities of interacting symbionts to promote the evolution of defense (Kerr et al. 2002; Kirkup and Riley 2004). On the other hand, diverse communities of interacting symbionts generate the same conditions under which more virulent symbionts are expected to evolve (Frank 1996). Consequently, models regarding the evolution of defense predict that co-infection of the same or different symbiont species, will cause selection for defensive mutualists (Jones et al. 2007; Fenton et al. 2011) while models regarding the evolution of virulence predict the evolution of virulent pathogens (Mosquera and Adler 1998) under the same conditions.

We develop a model with the goal of reconciling the conflicting model predictions of damaging pathogens and defensive mutualists under the same conditions of multiple symbiont infections. First, we construct a model in which the direct effects of a

symbiont on host mortality (virulence) range from positive to negative values. The direct effects on host mortality produce a feedback on the symbiont, altering its mortality rate. The model is similar to that of West et al. (2002) in that it focuses explicitly on symbiont mortality and reproduction in lieu of focusing on the host population. Second, we expand the model to encompass multiple infections by allowing the costs and benefits of symbiont infection to be shared by co-infecting symbionts, similar to varying Hamilton's r as shown in West et al. (2002). Last, we incorporate symbiont-mediated host defense by allowing symbionts to invest in traits that decrease the shared costs associated with co-infection. Throughout we examine the effects of defensive traits on symbiont evolution to determine whether host defense by symbionts can help resolve the paradox that we observe largely benign symbiotic communities while models predict increased virulence under multiple infections.

Methods:

Box 1: Terms used.

a_b: Slope of the number of reproductive propagules produced per increase in symbiont mortality

a_m: Slope of the increase in symbiont mortality per number of reproductive propagules produced.

$b(v,f)$: Rate of production of infectious propagules, a function of a symbionts harm to its host and its investment in defense

b_0: Basal rate of propagules production when virulence is zero

$c(I/N)$: Contact rate with susceptible host sites, a function of infection frequency

f: Defense, decreases the shared costs of virulence at a reproductive cost

g_b: Slope of the decrease in the shared costs of virulence to symbiont reproduction due to defense

g_m: Slope of the decrease in the shared costs of virulence to symbiont mortality due to defense

h_b: Slope of symbiont reproduction rate per investment in defense

I_x: Number of infections of species x throughout the host population

$m(v,f)$: Mortality rate of symbionts, a function of symbiont's harm to the host and investment in defense

m_0: Basal mortality rate of symbionts when virulence is zero

N: Number of individual hosts in a population

p_b: Shared costs to symbiont reproduction associated with co-infection

p_m: Shared costs to symbiont mortality associated with co-infection

v: Virulence, or increased host mortality due to symbiont infection and growth in the host

s: average number of symbionts per host

In this work, we focus explicitly on the status and fitness of symbionts. Our approach contrasts with the many models that track changes of the infection status of the host population to understand symbiont evolution (e.g. Bremmerman 1983; Nowak and

May 1994; van Baalen and Sabelis 1995; May and Nowak 1995; Mosquera and Adler 1998; Alizon et al. 2008; Jones et al 2011). We track the number of infections of each symbiont species, I_x and allow that there may be more than one infection per host. In our model infection success is a function symbiont infection frequency and not affected by absolute host population size, *per se*. Additionally, because this analysis focuses on symbiont virulence at equilibrium, the absolute size of host population has no effect on the results, as shown in Chapter 1, and is not explicitly defined in this model. Infections "die" within hosts at rate *m* infections per unit time and produce infectious propagules at rate *b* per unit time. These propagules transmit between hosts and establish new infections at rate *c*, making *bc* the rate of total effective symbiont transmission. Using this framework, we can distinguish symbiont processes occurring within a host population (*b* and *m*) from processes occurring between hosts (*c*). Together, the functions b, c, and m yield the expected change in number of infections for each strain *i* of symbiont species *x* (I_{ix}):

$$\frac{dI_{ix}}{dt} = I_{ix}(b_{ix}c_x - m_{ix}) \tag{1}$$

Where:

$$b_{ix} = \left(a_{bx}v_{ix} + p_{bx}\frac{1}{L}\left(\sum_j^L I_j\overline{v_j}\right)(1 + g_{bx}f_{ix})^{-1} - h_{bx}f_{ix} + b_{0x}\right)^B \tag{2.1}$$

$$m_{ix} = \left(a_{mx}v_{ix} + p_{mx}\frac{1}{L}\left(\sum_j^L I_j\overline{v_j}\right)(1 + g_{mx}f_{ix})^{-1} + m_{0x}\right)^M \tag{2.2}$$

$$c_x = 1 - \frac{I_x}{sN} \tag{2.3}$$

First, starting with single infections, we incorporate basal rates of propagule production and mortality, b_0 and m_0, respectively. The parameters, b_0 and m_0, give the rates of reproduction and death when a symbiont is commensal (v=0) and exhibits no defensive traits (f=0). Next we incorporate a tradeoff which increases both reproduction (b_x) and mortality (m_x) by incorporating the genetically variable trait virulence, v, into each term. In order to model symbiont interactions, we are translating virulence, typically defined as symbiont-induced host mortality, to symbiont mortality in order to track the costs of virulence between symbionts. The total costs and benefits of virulence are determined by the coefficients, a_{mx} and a_{bx}, which modulate the effects of virulence on symbiont mortality respectively. Here $a_{mx}v$ and $a_{bx}v$ represent the costs and benefits of virulence to a single symbiont genotype.

We next incorporate the indirect effects of a symbiont on its host by incorporating terms for shared costs under multiple infections and for defense of the host. Instead of focusing on specific mechanisms by which symbionts interact, we use the parameters p_b and p_m to encapsulate the shared costs associated with co-infection to symbiont reproduction and mortality, respectively. The shared costs of co-infection (p_b and p_m) include the maximum number of co-infections per host and the degree to which co-infecting symbionts interact and affect each other's reproduction or mortality. If both p_b and p_m are zero, symbionts have no effect on each other, and the model becomes a single infection system. If p_b is positive, co-infecting symbionts compete for resources and one symbiont can affect reproduction in another symbiont. If p_m is positive, symbionts affect each other's mortality and highly virulent parasites increase the mortality rates of co-

infecting symbionts by increasing host mortality. The coefficients p_b and p_m are multiplied by the average virulence over all L symbiotic species infecting a host population, weighted by the infection frequency of each species to give the total effect that co-infecting symbionts have on each other's reproduction and mortality. It is important to note that we are assuming, for simplicity, an even distribution of symbionts across the host population. Therefore the virulence of one symbiont directly affects mortality of other symbionts on the same host.

Finally, we incorporate defense with both costs and benefits to the symbiont. The costs of defense are determined by the parameter h_b. If h_b is positive, defense comes at a metabolic cost which decreases symbiont reproduction. Defense of the host indirectly benefits the defending symbiont by decreasing the costs associated with a co-infecting deleterious parasite. Therefore, we divide p_b and p_m by values for the strength of the defensive trait, multiplied by a coefficient g_b and g_m, plus one. If both g_b and g_m are zero, investment in defense yields no benefit and defense is not evolvable. It is important to note that we are focusing on the effects of defense on the shared costs of virulence. We are neglecting the effects that direct interference competition between symbionts may have on these symbionts' reproduction, mortality, or infectivity.

We complete expressions for b and m by raising the sum of the terms described above to the powers B_x and M_x, respectively. By making $M_x > B_x$, mortality will grow faster than reproduction as either v or f increase ($m'>b'$), allowing an evolutionary stable state to arise where:

$$c_x = \frac{m_x'}{b_x'} \tag{3}$$

Because we use a simple logistic function for the per-symbiont rate of establishing new infections (c), the probability of a new infection decreases as the number of current infections increases. Thus, Equation 1 produces a negative density-dependent feedback similar to that in (Lenski and May 1994). This negative density dependent feedback results in an ecological equilibrium where:

$$c_x = \frac{m_x}{b_x} \tag{4}$$

Therefore the system reaches an ecological and evolutionary equilibrium for each trait where:

$$\frac{m_x}{b_x} = \frac{m_x'}{b_x'} \tag{5}$$

Finding equilibria:

As closed form solutions can only be expressed under single infections, we used a search algorithm to find evolutionary stable state (ESS) solutions for the model under multiple infections. Initial values of virulence, defense, and infection frequency were chosen (v_x=0.1, I_x=0.1, f_x=0) corresponding to a pathogenic symbiont with no investment in defense at an initially low infection frequency. We initialize symbionts as pathogenic to provide a conservative view on the evolution of mutualism. Thus, we model the evolutionary forces that result in transitions from pathogenism to mutualism, as opposed to simply modeling the maintenance of mutualism. Assuming a constant supply of

genetic variation, we calculate selection coefficients for each trait and then, determine mean trait values in the next generation. A new infection frequency is then calculated using Equation 1. The process is repeated until virulence, defense, and infection frequency reach an equilibrium. A limitation of this approach is that as infection approaches one, *c*, as defined by Equation 2.3, may approach zero and thus limit selection on traits affecting *b* before the evolutionarily stable state is reached. To avoid this artifact and allow traits to reach evolutionary equilibrium, infection frequency is capped at a number close to, but not equal to, one (0.9995 for the results presented here).

Results:

We examine the impact of multiple infections within a symbiont species and then between two species on the evolution of virulence, and do so with and without allowing the evolution of defense. One species in the model has a positive basal rate of propagule production ($b_0 > 0$) and has the potential to act as a mutualist by decreasing the host mortality rate (negative virulence) The second species has a no basal reproduction ($b_0 = 0$) and is an "obligate pathogen" that must increase host mortality rate to reproduce (positive virulence). The effect of each symbiont on other co-infecting symbionts is modeled through the shared costs of host mortality as this affects the mortality or

reproduction of all resident symbionts.

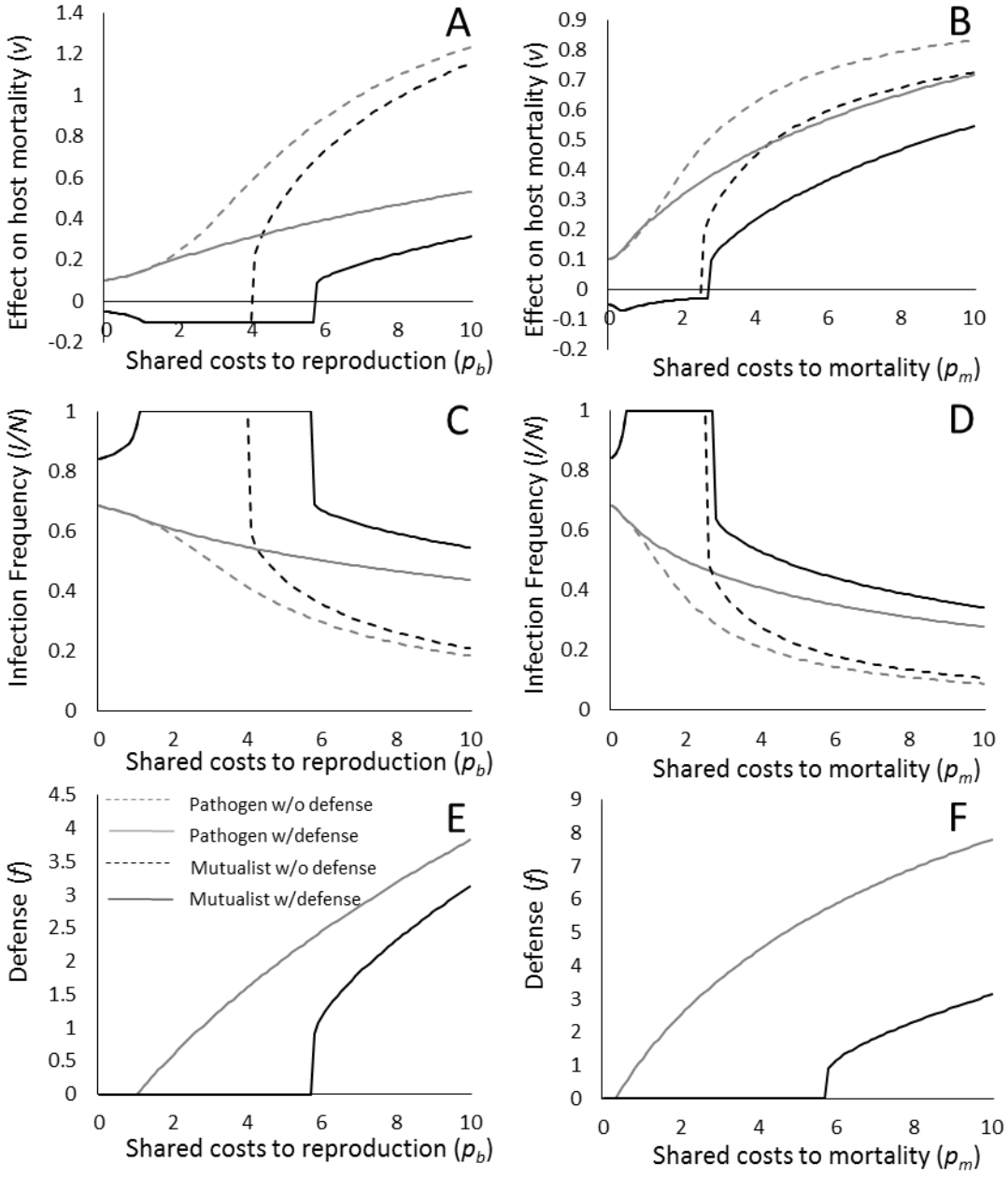

Figure 2.1: Evolutionary stable states under intraspecific shared costs as determined by Equations 1 and 2.1 through 2.3. Direct effect of a symbiont on host mortality (A and B), infection frequency (C and D), and evolved defense (E and F) at equilibrium

are shown for increasing values of shared costs to symbiont reproduction (p_b; A, C, E) and symbiont mortality (p_m; B, D, F). The costs of co-infection are shared within species for either an obligate pathogen (grey, b_0=0) or a potential mutualist (black, b_0=0.3). Evolutionary stable states without evolvable defense (g_m=0 and g_b=0) are shown with dashed lines, and evolutionary stable states with evolvable defense (g_m =1 or g_b =1) are shown with solid lines.

Intraspecific shared costs

The level of shared costs affects both the evolution of virulence (Figure 2.1 A, B) and symbiont infection frequency (I/N; Figure 2.1 C, D) at equilibrium. Panel A shows the evolution of virulence in an obligate pathogen (dashed grey) and a potential mutualist (dashed black) when shared costs affect symbiont reproduction. In Figure 2.1 A, results show that when shared costs are low, the potential mutualist (black line) evolves negative virulence, indicating a mutualistic relationship with the host. When shared costs are high, mutualism is lost and the potential mutualist evolves positive virulence or parasitism. Similarly, as shared costs increase among multiple infections of a pathogenic species (dashed grey line), more damaging symbionts evolve. Similar results for the evolution of virulence are obtained when shared costs affect symbiont mortality rather than reproduction (Fig. 2.1 B). When shared costs are low, the potential mutualist evolves negative virulence. Conversely, when shared costs are high, both the potential mutualist and the obligate pathogen evolve higher virulence. However, as shared costs increase, the

magnitude of the increase in virulence is much smaller when shared costs affect mortality than when they affect reproduction.

Virulence has costs for population size as well (Fig. 2.1 C, D). In the obligate pathogen (dashed grey lines) higher shared costs to symbiont reproduction always lead to lower infection frequencies. However, in the potential mutualist, low to moderate shared costs actually lead to increased infection frequencies, as co-infecting mutualists increase each other's transmission rates by decreasing host mortality. Similar results are obtained if shared costs affect symbiont mortality; the obligate pathogen suffers lower infection frequencies and the potential mutualist may increase infection rates at low to moderate shared costs. By comparing Figure 2.1 panels C and D, one can see that infection frequencies decrease faster as shared costs increase when shared costs affect mortality (panel C) than when shared costs affect reproduction (panel D). This result is surprising given that, as mentioned above, virulence increases more slowly when shared costs affect mortality than when shared costs affect reproduction. However, a central assumption of the virulence tradeoff hypothesis is that mortality increases faster than transmission as virulence increases. Therefore, an increase in shared costs to symbiont mortality has a greater impact on infection frequency than the same change in the shared costs to reproduction.

By comparing the evolution of virulence with defense (solid lines Figure 2.1 A, B) to the evolution of virulence without defense (dashed lines Figure 2.1 A, B), we show that evolvable defense tempers the effects of shared costs on both the potential mutualist

and the pathogen. Figure 2.1 E shows that pathogenic species (grey line) invest in defense at relatively low levels of shared costs while shared costs must be large before the potential mutualist (black line) invests in defense. This investment in defense in turn leads to the evolution of lower virulence in both species than when defense is not available. In the potential mutualist (Figure 2.1 A, B, black lines), the evolution of defense maintains mutualisms at higher shared costs than without defense. As shared costs continue to increase, defense allows the evolution of less damaging parasites even after the symbiont switches to a pathogenic strategy ($v > 0$). It is important to note that an exception to the rule that defense leads to lower virulence arises when shared costs affect symbiont mortality. When shared costs to mortality are low, evolvable defense actually leads to slightly more damaging pathogens (Figure 2.1 B, grey lines, $p_m < 1$). This unexpected result will be explored further below.

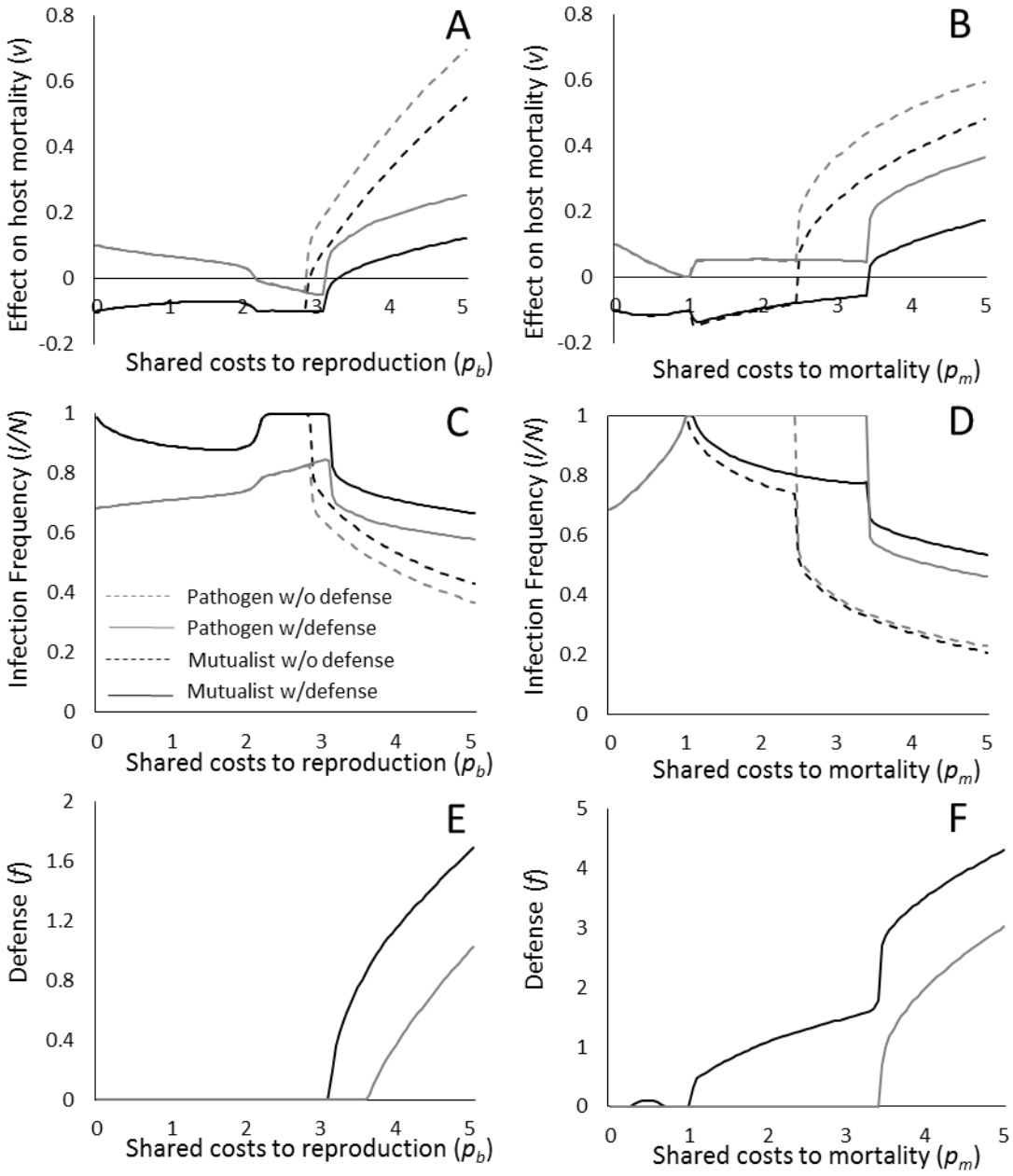

Figure 2.2: Evolutionary stable states under interspecific shared costs.

Direct effect of a symbiont on host mortality (A and B), infection frequency (C and D), and evolved defense (E and F) at equilibrium are shown for increasing values of shared costs to reproduction (p_b; A, C, E) and mortality (p_m; B, D, F). The costs of

co-infection are shared within an obligate pathogen (grey, b_0=0) or a potential mutualist (black, b_0=0.3). Evolutionary stable states without evolvable defense (g_m=0 and g_b=0) are shown with dashed lines, and evolutionary stable states with evolvable defense (g_m=1 or g_b=1) are shown with solid lines.

Interspecific shared costs.

When costs are shared between symbiont species, both evolutionary processes within species and co-evolutionary processes between species may result. To compare the effect of interactions within species with interactions between species, we next examine the effects of multiple infection when the costs and benefits of virulence are shared between, but not within species. When costs are shared between species, the presence of a mutualist can lead to the evolution of decreased virulence in a pathogen.

Figure 2.2 A shows the evolution of virulence when intraspecific shared costs affect symbiont reproduction. Here, at low to moderate levels of shared costs, the presence of a mutualist species increases the reproductive rate of the pathogen, facilitating the evolution of lower virulence and even mutualism in the erstwhile pathogen. When shared costs are high, the potential mutualist evolves positive virulence, resulting in increased virulence in both species. Figure 2.2 B shows the evolution of virulence when intraspecific shared costs affect symbiont mortality. Again, the presence of a mutualist leads to the evolution of decreased virulence in the pathogen and can even

lead to commensalism in the pathogen (v=0). In contrast to Figure 2.2 A, when shared costs affect mortality, the erstwhile pathogen never evolves mutualism.

Shared costs also affect infection rates of pathogens and mutualists differently (Figure 2.2 C, D). Results show that low to moderate shared costs to reproduction between a pathogen and a mutualist can result in increased infection frequencies (I/N) for the pathogen but decreased infection frequencies for the mutualist. When shared costs are high, both species suffer lower infection frequencies. Figure 2.2 D shows the effect of shared costs between species on infection frequency when shared costs affect mortality. When shared costs are low, co-infection with a mutualist (dashed black) leads to increasing infection frequencies in the pathogen (dashed grey). As shared costs increase, the deleterious effects of the pathogen lead to decreasing infection frequencies in the mutualist. Finally, when shared costs are high, infection frequencies in both species decrease.

Figure 2.2 shows results comparing the evolution of virulence with defense (solid lines) and the evolution of virulence without defense (dashed lines). We show that evolvable defense leads to lower virulence in both the potential mutualist and the pathogen as shared costs increase. Additionally, Figure 2.2 A and B show that defense results in the evolution of mutualism at higher shared costs than when defense is absent. Figure 2.2 E shows that when shared costs are low and affect symbiont reproduction, neither the mutualist nor the pathogen invest in defense. As shared costs increase the mutualist invests in defense, and when shared costs are high the pathogen invests in defense. In contrast, Figure 2.2 F shows that, when shared costs affect mortality, the

mutualist invests in defense even at fairly low levels of shared costs. When shared costs are high, both the pathogen and the potential mutualist invest increasingly more defense.

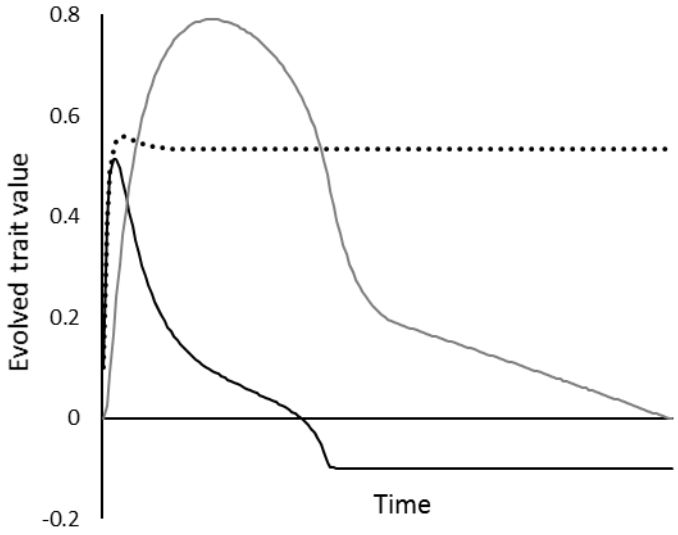

Figure 2.3: The evolution of mutualism and defense over time with shared costs affecting symbiont reproduction. To show how defense can facilitate the evolution of mutualism and then be lost, a time series shows the evolution of virulence in a potential mutualist (b_0=0.3; p_b=5) without defense (g_m=0, dotted line) and with defense (g_m =1, solid line), and the level of evolved defense (grey). The parameters used were: a_b=4, a_m=1, m_0=0.1; h_b=0.1.

Effects of evolvable defense on mutualism

The results of intra- and interspecific shared costs shown above yield two unexpected outcomes. First, under intraspecific shared costs, evolvable defense facilitates the evolution of mutualism even though investment in defense is zero at equilibrium (Figure 2.2 A, E; black line where $4<p_b<5.8$). Second, when shared costs affect mortality, there is a small region where symbionts are more virulent with evolvable defense than without. This raises the possibility that under certain conditions, evolvable defense might lead to more damaging parasites and actually disrupt mutualisms.

Figure 2.3 shows a time series of the evolved levels of virulence to the host and for investment in defense, with a potential mutualist under a single value of shared costs (p_b =5). Starting from a pathogenic, low infection frequency state, selection favors more damaging strains. Without evolvable defense, harm to the host plateaus and the potential mutualist evolves to a steady state of positive virulence, it is a parasite. In contrast, with evolvable defense, both the level of harm to the host and defense increase in concert, peak, and then decrease together. Once the population reaches a mutualistic state, defense evolves to zero. Therefore, defense can facilitate the evolution of mutualisms, and in this simple single symbiont system, defense will not be maintained as the mutualism stabilizes.

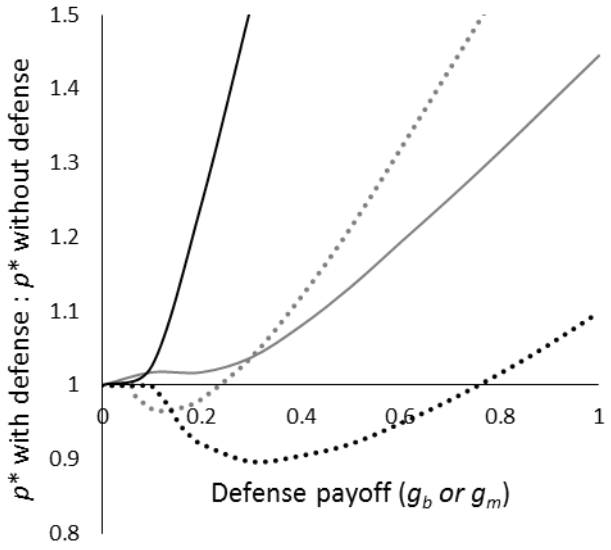

Figure 2.4: Defense payoff and the evolution of mutualism. To investigate the effect of return on investment in defense (g_m and g_b) on the critical value of shared costs at which a potential mutualist evolves parasitism (p^*), we examine the ratio of p^* with defense to p^* without defense. Values above one indicate that evolvable defense facilitates mutualism, values below one indicate that evolvable defense hampers the evolution of mutualism. Four scenarios of shared costs are shown, shared costs within a species of mutualists (black), between a potential mutualist and an obligate pathogen (grey), shared costs affecting reproduction are shown in solid lines, and shared costs affecting mortality are shown in dotted lines. Unless otherwise specified a_b=4, a_m=1, m_0=0.1; h_b=0.1.

To determine if defense always facilitates mutualism, we broaden our approach and examine a range of payoffs for investment in defense. Thus far we have examined a single payoff regime where the costs of defense are relatively low compared to the benefits (h_b=0.1, g_m =1 or g_b = 1). Because we are primarily interested in the effect of evolvable defense on the evolution of beneficial symbionts, we focus on the critical value of shared costs at which the potential mutualist evolves to a pathogenic state, which we call p^*. In Figure 2.1A, for example, $p^* \approx 5.8$ with defense and $p^* \approx 4$ without defense. To illustrate how the costs of a payoff of investment in defense affects the evolution of mutualism, Figure 2.4 shows the ratio of p^* with and without evolvable defense as costs of defense increase. Ratios of p^* with and without evolvable defense greater than one indicate that the defense tradeoff allows mutualism to evolve at higher levels of shared costs. Values less than one indicate that evolvable defense can actually disrupt mutualisms and select for parasites. When shared costs affect reproduction, defense always preserves mutualisms. However, when shared costs affect mortality and g_m is low, the defense tradeoff can actually disrupt mutualisms under both intraspecific and interspecific shared costs and lead to parasitism.

Discussion:

Many organisms are host to symbionts that protect them from biotic enemies (e.g., Arnold et al. 2003; Oliver et al. 2005; Gast et al. 2009; Jaenike et al. 2010; Pringle 2014). Vertically transmitted symbionts exhibiting low virulence have generally been the

focus of research concerning symbiont defense of the host (Lively et al. 2005; Haine 2008; Jones et al. 2011). However, the realm of defensive symbionts is not limited to vertically transmitted mutualists. Pathogens may also inhibit competing pathogens (Fisher and Mayor 1986; Gardner et al. 2004; Balmer et al. 2009) and some defensive symbionts are horizontally transmitted (Kaltenpoth and Engl 2014). In this work, we put forth a model that encompasses the evolution of defense in both parasites and mutualists with horizontal transmission. Symbionts affect each other through the shared costs of virulence as effects on host mortality that indirectly affect symbiont mortality or reproduction. We focus our analysis on the interplay between the evolution of defense and the evolution and maintenance of mutualism. Finally, we discuss some key assumptions underlying the model to point the way towards future research.

Many models for the evolution of defensive symbioses have assumed single infections within species (Lively et al. 2005; Sorrell et al. 2009), no variation in the level of protection provided by a defensive symbiont (Heithaus et al. 1980; Lively et al. 2005; Sorrell et al. 2009; Fenton 2011; Kwiatkowski and Vorburger 2012), and vertical transmission of the defensive symbiont (Jones et al. 2011; Jones et al. 2012). We expand upon this body of literature in three ways. First, we utilize the concept of shared costs of virulence developed in Chapter 1 to model multiple infection both within and between symbiotic species. Second, we expand on Fenton (2011) and Kwiatkowski and Vorburger (2012) by modeling the simultaneous evolution of two quantitative traits, symbiont virulence and symbiont defense of the host. In our model, the level of protection a symbiont provides its host is variable and subject to selection, allowing us better model

the role of natural selection in maintaining defensive mutualisms (Johnson et al. 1997; Nelson and May 2014. Third, we generalize Jones et al. (2011) and Jones et al. (2012) by incorporating horizontal transmission between hosts. Thus symbionts in our model can transmit horizontally and establish multiple infections, both of different genotypes within species and of different species. Our model synthesizes the evolution of defensive symbioses and the evolution of virulence to better reflect processes within the complex symbiotic communities observed in nature.

Like established models such as Lively et al. (2005), Sorrell et al. (2009), and Jones (2011), our model seeks to understand how interactions between symbionts affects the evolution of virulence towards the host. Similar to our model, Jones et al. (2011) treats multiple infections through the shared costs of virulence. In both our model and Jones et al. (2011), defense allows symbionts to avoid shared costs associated with co-infection. Consequently, both models concur with the empirical findings that defending a host can provide a selective advantage for symbionts (Jaenike et al. 2010; Fenton et al. 2011). However, where Jones et al. (2011) assumes single infections within species, we model the effects of multiple infections on a continuum allowing for multiple infections both within and between species. Additionally, in Jones et al. (2011), defense explicitly prevents infection by parasites, whereas in our model we do not specify the mechanism by which defense decreases shared costs. Instead, we focus on outcomes and are able to show that defense is unlikely to evolve when interactions between symbionts are weak or multiple infections are rare.

Because models with vertical transmission are expected to lead to the evolution of benign, if not beneficial symbionts even without defensive traits (Lipsitch et al. 1996), previous models (Lively et al. 2005, Sorrell et al. 2009, and Jones et al. 2011) have examined the evolution low or avirulent defensive symbionts, neglecting the possibility of defensive pathogens. In our model we make no assumptions regarding vertical or horizontal transmission. This added flexibility allows us to show that defense of the host can evolve in parasitic symbionts, especially under multiple infections by different strains of the same symbiont species. Additionally, we show that defense can lead to the evolution of mutualism under levels of shared costs that would otherwise lead to parasitism. Under intraspecific shared costs, we found that a transient investment in evolvable defense can facilitate mutualism in symbionts, despite little investment in defense at equilibrium. This counterintuitive result stems from the fact that virulent co-infectors are required to maintain selection for defensive traits, while in turn defense selects for less damaging symbionts. As defense selects for less damaging strains, selection pressure on defense relaxes. Thus, defense allows for the evolution of beneficial symbionts, at which point selection purges the defensive trait. This result raises the possibility that transient periods of investment in defense may leave a legacy of mutualism behind.

While our model shows that symbiont defense of the host can lead to lower virulence, by treating the shared costs of virulence as a quantitative trait, we find the counterintuitive result that defense can sometimes lead to the evolution of higher virulence in defending symbionts. Previous studies have shown that factors affecting

interactions between symbionts, such as relatedness and kin selection, can affect the outcome of virulence evolution under multiple infections (Koskella et al. 2006; Buckling and Brockhurst 2008). Whereas Koskella et al. (2006) and Buckling and Brockhurst (2008) invoke kin selection to explain the evolution of lower virulence, we show that symbiont defense of the host can also select for lower virulence. In our model the effect of a defense tradeoff on the evolution of virulence is dependent on the costs and benefits of defense. If symbiont investment in defensive traits entails little reproductive cost to the symbiont, the evolution of defensive traits lead to decreased virulence under multiple infections. However, if defense is costly, symbionts may compensate for the cost of investing in defense by harvesting more resources from their hosts, leading to an overall more pathogenic community than would be expected if defensive traits had not evolved. Thus, our model returns a result similar to Neuhauser and Fargione (2004) where the presence of a seemingly mutualistic symbiont can paradoxically lead to lower host fitness than if that symbiont were absent.

Future directions

In examining these tradeoffs we have made a number of simplifying assumptions. Chiefly, we have neglected the origin of novel genetic traits required for the evolution of virulence and defense. We have also neglected non-deterministic elements, such as genetic drift or environmental disturbances, which will dominate when selection pressures or population sizes are small or populations are spatially structured (Prado and Kerr 2008; Nahum, et al. 2011; Verbruggen et al. 2012). Similar models addressing

evolution of the host symbiont relationship have shown that, given sufficient genetic variation, a species may evolve to parasitism or mutualism depending on the initial conditions (Grover and Wang 2014; Chapter 1 of this thesis). Therefore, non-deterministic elements may be key in navigating between parasitic and mutualistic equilibria and are prime targets for future analyses.

We have also assumed no evolution on the part of the host. Host evolution is likely a key factor in the evolution of defensive symbioses and could interact with symbiont evolution in several ways. Hosts may evolve to minimize the costs associated with hosting defensive symbionts by evolving tolerance (Frederickson et al. 2012), resulting in lower virulence at no cost to the symbiont. Symbiotic defense of the host could also release a host from pressure to defend against enemies (Nomura et al. 2011). Additionally, diversity within the symbiotic community could lead to polymorphisms within the host population (Heil et al. 2009) in host traits such as investment in defense and tolerance of symbionts. As we have shown that evolution of just two traits in a symbiont can produce counterintuitive results, the outcomes of coevolution between hosts and defensive symbionts is likely to be particularly hard to predict. Including host evolution may further elucidate the mechanisms by which benign symbioses are maintained in symbiotic communities.

Conclusion

The ubiquity of seemingly harmless, and even beneficial symbionts infecting plants and animals is one of the greatest puzzles facing evolutionary biology. We have

put forth a model in which symbionts can affect their hosts in two ways, directly through the effect on host mortality, and indirectly through defending the host against other symbionts. We have demonstrated that defense largely leads to less damaging pathogens and preserves mutualisms under co-infections. However, the availability of a defense tradeoff can lead to more deleterious pathogens and disrupt mutualisms if the costs and benefits affect different life history stages of the symbionts. Thus, while defensive traits in symbionts may be key to maintaining benign symbioses, understanding how defensive traits affect the host and symbiont is vital to understanding the evolution of the host symbiont relationship.

Chapter 3: Genetic variation for pathogen virulence and interactions with a defensive symbiont of maize.

ABSTRACT

In Chapters 1 and 2 of this thesis we model the selective forces that shape the evolution of symbiont virulence and defense of a host. In this chapter, we determine evidence for genetic variation for in pathogen and symbiont populations. We used a model system of maize, a pathogen *Ustilago maydis*, and a benign symbiont, *Fusarium verticillioides* to determine evidence for variation in virulence by the pathogen, and defensive traits of the symbiont. We found that *U. maydis* strains from two populations exhibited significantly different levels of virulence towards the host. In addition, we found evidence for genetic variation in *F. verticillioides* populations for antagonism toward *U. maydis*, a defensive trait. Contrary to expectations, we found that *U. maydis* growth was enhanced by *F. verticillioides*, suggesting that *F. verticilloides* may sometimes facilitate, rather than antagonize, pathogen growth. Because both symbionts have free-living life history stages, we evaluated evidence for pleiotropy in *U. maydis* between virulence toward the host and growth as a saprophyte. Results showed a negative correlation between virulence and growth, suggesting that countervailing selection may act on these traits.

Introduction:

Fungal symbionts commonly occur in plants and have a range of effects on host fitness from pathogens that harm their hosts, to mutualistic symbionts involved in nutrient acquisition (Mazancourt and Schwartz 2010), environmental tolerance (Márquez et al. 2007) or protection against biotic enemies (Arnold et al. 2003). The body of theory addressing symbiont evolution suggests that host genotype plays an important role in the evolution of symbiont traits (McLean, 1995; Gupta and Anderson 1999; Magori and Park 2014). Indeed, many studies have found evidence of genetic variation in symbiont species both within (Oono et al. 2014) and between host populations (Capelle and Neema 2005; Heath and Tiffin 2007; Johnson et al. 2010; Covarelli et al. 2012). Disease causing symbionts, in particular, show population level differences in traits such as infectivity and virulence (Thrall and Burdon 2003; Boots et al. 2004; Fischer and Foitzik 2004; Springer 2007; Alshareef and Robson 2014; Bruns et al. 2014; Stefansson et al. 2014; Voyles et al. 2014). Throughout this paper we define virulence as symbiont induced harm to host growth. Here we examine evidence for genetic variation in virulence in populations of a pathogen and of defensive traits in a benign symbiont of plants to determine evidence for microbial interactions causing selection on virulence and defense traits.

While previous studies have shown population level variation in pathogen virulence (Kniskern et al 2007; Pan et al. 2008; Carvalhais et al. 2013), less is known about variation in traits that affect interactions between symbionts (May and Nelson 2014). Co-infecting symbionts interact through mechanisms such as excreted metabolites, effects on host gene expression, alterations of host physiology, or effects on host

mortality rates (Saunders et al. 2010; Laine 2011; Ferrari and Vavre 2011; Larimer, 2012; Meija et al. 2014; Panaccione et al. 2014). Interactions between symbionts can range from facilitation (Cattadori et al. 2008) to inhibition and affect the infection, growth, or reproduction of co-occurring symbionts (Borowicz 2001; Al-Naimi et al. 2005). Of particular interest are defensive symbionts that inhibit pathogens, thereby protecting their host from enemies (Jaenike and Brekke 2011). Indeed, theory predicts that the presence of a virulent pathogen may cause selection for symbionts that may prolong their own infection growth in the host by inhibiting pathogens that harm the host (Thompson et al. 2002; Jones et al 2011; Chapter 2 of this dissertation). In this study, we examine evidence population level variation in virulence by an important pathogen of maize, *Ustilago maydis.* To determine evidence for genetic variation in symbiont mediated host-defensive traits, we examine inhibition of different *U. maydis* genotypes by a common symbiont of maize, *Fusarium verticillioides*.

This study focuses on interactions of two common fungal symbionts of maize, the pathogen *U. maydis* and the endophyte *F. verticillioides.* The basidiomycete *U. maydis* causes damaging infections in stem, ears, anther, and leaf tissues of maize and its wild relative teosinte (Bölker 2001). Additionally, *U. maydis* alters the metabolism of infected tissue, changing infected leaves from a carbon source for the plant into a carbon sink (Doehlemann et al. 2008; Horst et al 2010). While genotypes of *F. verticillioides* may cause disease in ears (Desjardins and Plattner 2000), other genotypes grow asymptomatically in other tissues as endophytes (Pan et al. 2008; Lee et al. 2009; Pan and May 2009; Saunders and Kohn 2009). Importantly for this study, *F. verticillioides* acts as

a defensive symbiont by inhibiting *U. maydis* growth, thereby decreasing damage done to the host (Lee et al. 2009). In addition to the infectious life history stages in maize, both fungi have free living stages in soil or plant debri (Bölker 2001; Cavaglieri et al. 2005; Vollmeister et al. 2011; Funnell-Harris and Pedersen 2011), making them ideal for studying microbe-microbe interactions with potential impacts on host fitness. Moreover, *F. verticillioides* detoxifies the host defense compound 2-benzoxazolinone (BOA) (Richardson and Bacon 1995; Cambier et al. 2000; Glenn et al. 2002; Saunders and Kohn 2009), and in doing so, facilitates infection of maize by diverse fungi (Glenn et al 2001; Saunders and Kohn 2009). BOA is especially likely to affect interactions between these two fungi given that both *F. verticillioides* and *U. maydis* are sensitive to BOA but *F. verticillioides* degrades BOA (Glenn et al. 2002). Consequently, we can use *in vitro* assays with BOA to determine if the outcomes of symbiont interactions are affected by the host context.

The presence of a virulent pathogen like *U. maydis* should put selection pressure on other symbionts, such as *F. verticillioides*, to invest in defensive traits that lead to inhibition of those pathogen's infection or growth within the host (Thompson et al. 2002; Jones et al 2011; Chapter 2). To measure variation in antagonism between these two species as a proxy for a defensive trait, we paired *U. maydis* and *F. verticillioides* strains *in vitro* and compared colony growth rates of each organism alone and in co-culture. We evaluated whether the presence of BOA, a maize defensive compound, alters outcomes of these *U. maydis* and *F. verticillioides* interactions. To determine if interactions of these two fungal symbionts of maize might cause selection for increased antagonistic traits, we

used the results of a field study to evaluate correlations between co-occurrence and antagonism. Finally, because *U. maydis* has both parasitic and saprophytic phases, selection on life history traits important for the saprophytic phase may constrain the evolution of virulence towards the host. Therefore, we compared the growth of *U. maydis* strains *in vitro* with virulence *in vivo* to determine evidence that tradeoffs between saprotrophic and symbiotic traits constrain the evolution of virulence.

Methods

Fungal strain isolation and collections

We sought fields that had been continuously planted with resistant maize or susceptible maize, and thus provide differing selection on *U. maydis* virulence, by consulting with seed providers and farmers in southern MN and the UM field station on the St. Paul campus. We sampled from one field that has been historically planted with disease resistant, commercial, hybrid maize (Field corn), and the other with a disease susceptible, inbred variety (W22) commonly used in research (Candela and Hake 2008; Santiago et al. 2013). Plants in both fields were sampled along the natural transects of planted rows for both *U. maydis* and endophytic fungi. In each field, we assessed the spatial distribution of *U. maydis* and determined the rate of co-occurrence of the two fungal species by sampling two adjacent plants at each of 20 sampling locations, one with a *U. maydis* gall and an adjacent plant without obvious *U. maydis* infection. For the plant with the *U. maydis* gall, the gall containing *U. maydis* spores was removed to a sterile 50

mL Falcon tube and an approximate 1 cm square tissue sample was taken from each of four cardinal points approximately 1 cm from the gall location. From the adjacent plant without *U. maydis*, four similar samples were taken from similar locations in the plant tissue.

In the Field corn plot, the frequency of *U. maydis* galls was very low (ca. 1.4 %). Plants were examined exhaustively and each plant located with a *U. maydis* gall was sampled along with an adjacent, uninfected plant as above. In the W22 plot, disease incidence was very high (ca. 76%) and we searched for plants that showed no symptoms of *U. maydis* disease and then sampled the adjacent plant with *U. maydis* galls. The spatial distribution of sampling points in the two fields was made similar by pacing approximately the same distance between uninfected plants in the W22 field as that of infected plants in the Field corn.

Endophyte isolation

Most samples were removed from stem tissue because *U. maydis* galls occur there most frequently. Falcon tubes containing sampled tissue segments were placed on wet ice in the field and returned to the lab. Within 24 hours of collection, tissue samples were surface sterilized by rinsing with sterile, deionized water, submerging in 10% EtOH for 1 min, 10% bleach for 30 seconds, 10% EtOH for one minute, and rinsing again in sterile water (Arnold et al., 2003). Each surface sterilized tissue segment was then split in half with half retained at -80C for metagenomic analyses, and the remaining half placed in antibiotic water agar with kanamycin (50 µg/mL)and ampicillin (100 µg/mL) (AWA).

Fungal colonies growing out from the tissues were then transferred to potato dextrose agar on 80mm plates and finally stored as water vouchers at room temperature. Water vouchers place small 1 cm^3 agar blocks with cultured fungi into small sealed tubes covered with sterile water. Fungal cultures can be stored for several years in this manner (McGinnis et al. 1974).

U. maydis isolation

Only the haploid yeast-like phase of *U. maydis* can be grown in axenic media whereas the dikaryotic phase (two n nuclei per cell) formed after mating of two compatible haploid yeasts, is obligately dependent on a living plant. Consequently, we obtained haploid cells and re-constituted dikaryon genotypes that represent each *U. maydis* gall. To obtain haploid yeast cells, *U. maydis* galls were crushed using mini pestles and the resulting diploid teliospores soaked in sterile 1% Copper (II) Sulfate for three days. Teliospores were then rinsed in distilled H_20 (diH_20) by suspension and centrifugation and spread on water agar amended with kanamycin (50 µg/mL) and ampicillin (100 µg/mL). Diploid telisospores germinate and produce haploid sporidia on the plate, and these haploid cells were collected and streaked on PDA plates (Zahiri et al. 2005) to obtain single spore isolates. Single spore colonies were then tested for mating compatibility on charcoal plates (as cited in Banuett and Herskowitz 1989). Compatible pairs were selected and each grown separately in 50mL liquid potato dextrose broth (PDB; BD Difco™) shaken at 150 rpm for 48 hours at 25C. Haploid cells were stored by

placing 1 mL of cultured cells (ca. 10^8 cells/mL) on sterile silica gels following the method of Perkins (1949).

Endophyte identification

We used DNA sequence and morphology to identify fungal endophytes emerging from the sampled maize tissues. Total DNA was extracted from mycelia in agar plugs using a Qiagen DNA extraction kit and the ITS-LSU region of the rDNA used to identify the fungi as previously (Pan et al. 2008; Pan and May 2009). Sequences were trimmed in Sequencher and BLAST results against Genbank were used for species identification. The endophyte *F. verticillioides* was classified as co-infecting with *U. maydis* if isolated from one of the four tissue segments cut from a plant with *U. maydis* galls. *U. maydis* strains were classified as co-infecting with *F. verticilloides* if one of the four tissue segments yielded *F. verticillioides.* It is important to note that co-infection status of *U. maydis* only refers to co-infection between *U. maydis* and *F. verticillioides* as all plants were host to multiple endophytes.

Virulence assay

To test for variation in virulence between *U. maydis* strains isolated from the two fields, we inoculated plants with compatible haploid pairs representing eleven dikaryon genotypes isolated from Field corn and twelve dikaryon genotypes from the W22 field (Table 3.2). Compatible *U. maydis* mating types grow as yeasts *in vitro* but form an infectious and filamentous dikaryon in the plant (Bölker 2001). Therefore, we assessed *U. maydis* virulence by inoculating the same two compatible haploid strains (dikaryon

type) into each plant with eleven replicate plants per treatment. As we explain below, we used the same compatible pairs or dikaryon types for interaction studies *in vitro*.

We used the size of inoculated plants as a proxy for virulence. We utilized Jubilee sweet corn, which is susceptible to both *U. maydis* and *F. verticillioides* infection. Seeds were planted 4 cm deep in pasteurized soil in conical pots 3.8 cm diameter wide and 21 cm deep and inoculated with *U. maydis* 8 days after planting. Inoculum was prepared by growing *U. maydis* haploid strains individually in PDB as described above for 48 hours. Haploid cells were concentrated and washed with sterile water. Cell concentration was then adjusted to 10^6 cell/ul as per (Rodriguez Estrada et al. 2012). Sporidia from each compatible mating type were mixed, and 80 uL of inoculum was pipetted into the whorl of each plant by pipette.

Greenhouse conditions and measurements of plant growth

Plants were grown under natural lighting and supplemental light intensities at 120–200 μEm^{-2}.with a 15h/9h light/dark cycle. Plants were watered every other day and kept between 24-30^{0}C at the University of Minnesota Plant Growth Facilities, St Paul.

Measuring plant size and virulence

To measure virulence, plant size was used as a proxy. To measure plant size, all plants were photographed three weeks after inoculation at two different angles from a fixed distance against a white cardboard background with a meter stick for scale. Photographs were cropped to be uniform and black areas were removed using GIMP to ensure that the plant was the only object visible in the image. Photographs were then

converted to binary black and white making the plant solid black against a uniform white background. The average black value of each photograph was recorded as a measure of plant size using ImageJ.

Experimental design to test fungal interactions

To determine if different *U. maydis* and *F. verticillioides* strains vary in their ability to inhibit each other's growth, we measured colony size of each species when grown with each other and compared that to control growth of the same colonies grown alone. To test for ecological factors associated with genetic variation in inhibition between species, we tested the effect of three factors on the colony size of each strain; the field from which a strain was isolated, the co-infection status at isolation, and the plant defense compound BOA on *in vitro* growth of each species. We used a balanced full factorial for a total of 16 strains for each fungal species (see Tables 3.1 and 3.2 for strain designations). To make the results of the *in vitro* experiment as comparable to the results of *in vivo* measurements, we mixed the same two compatible haploids from the same germinated teleospore to represent each *U. maydis* dikaryon strain for both sets of experiments. To measure colony growth in co-culture, each *U. maydis* strain was paired with each *F. verticillioides* strain on two replicate plates with BOA, and two without BOA, for a total of 1024 plates. Fungi were inoculated 4 cm away from each other in the paired plates. To measure growth alone, each strain from each species was grown in three plates with BOA and three plates without BOA, yielding three full experimental replicates for 96 plates. To test effects of BOA on fungal growth and interactions, fungi

were grown in 1% Potato dextrose agar with a final concentration of 0.5mg/ml BOA and 1% EtOH, or PDA amended with 1% EtOH as a control (Glenn et al. 2002). Plates were placed in a dark growth chamber at 22C. To ensure that *F. verticillioides* did not overgrow *U. maydis,* fungal growth was observed daily and the experiment has halted after the first *F. verticillioides* colony grew into the *U. maydis* colony. The first encounter between *F. verticillioides* colonies and *U. maydis* occurred after five days of growth, thus colony size was measured five days after inoculation.

Measurement of fungal growth in vitro

Inoculated petri plates were photographed at a fixed distance from camera to plate after five days of growth after inoculation. All image processing and analysis was performed in ImageJ (Schneider et al. 2012). Photographs were cropped to a uniform size and scaled to the diameter of the plate (90mm) to ensure consistent measurements. Each fungal colony was then outlined by hand and the area calculated automatically using the "Measure" function in ImageJ.

Statistical analysis

This study utilized three separate statistical tests to evaluate variation in virulence, interactions *in vitro* with and without BOA added to the media, and to determine the correlation of *U. maydis* strain virulence and colony growth. We used an ANOVA to test the effect of *U. maydis* field of origin and co-infection status on virulence towards the host. Field of origin was treated as a fixed effect and tested using the between strain

variance in virulence to determine if strains from the two fields exhibited significantly different levels of virulence. Plant size was used as an indirect measurement of virulence.

To investigate interactions between fungi in vitro, fungal growth was analyzed using a nested mixed linear model using the Lme4 package in R. We tested the effect of two strain factors: strain field of origin and strain co-infection status at isolation. We also tested the effect of strain field of origin and strain co-infection status of one fungal species on growth of the other species, which we call cross species factors. We also tested the effect of BOA on fungal growth, as well as statistical interactions between BOA, strain factors, and cross species factors.

This experiment used three different levels of variation as error: plate variation, within species strain variation, and variation in cross species strain by strain statistical interactions. Plate variation had the highest number of degrees of freedom, followed by variation in cross species strain by strain statistical interactions, leaving within species strain variation with the least degrees of freedom. Strain level variation was nested within the strain factors and cross species factors. Thus strain level variation was used to test the significance of strain field of origin and strain co-infection status on strain growth. We tested the effect of the plant defense compound BOA on fungal growth using between plate variation as error. Additionally, statistical interactions between BOA and strain effects, and BOA and cross species effects, were tested using strain level variation as error. Finally, statistical interactions between strain factors and cross species factors were tested using variation in strain by strain statistical interactions, as were all third and

higher order statistical interactions. Thus, the nested mixed linear analysis had the highest power in third order and higher level statistical interactions, which are difficult to interpret. Therefore, because we are primarily interested in the effects on fungal growth of BOA, field of origin, co-infection status, and interactions between these three main factors, we limited the analysis to first and second order statistical interactions. To evaluate first and second order effects the mixed model was run using the following code in R:

lmer(Farea~(BOA+Ffield+(1|FusStrain)+Ufield+(1|UstStrain)+Fco+Uco+Uarea)^2))

To test for significant differences between treatment means within statistical interactions, a Tukey HSD was performed on the linear mixed model for interactions that were significant in the full model.

To test for a correlation between *in vitro* growth and *in vivo* virulence, a parametric bootstrap was performed using parameters generated by the lmer function in R. The lmer function was used to determine strain means and variance for *U. maydis* growth while co-cultured with *F. verticillioides*, and for *U. maydis* virulence in maize. Due to the design of the experiment we had 64 observations per *U. maydis* dikaryon strain in competition with all *F. verticillioides* strains but only six observations per dikaryon strain of *U. maydis* grown in isolation. Therefore, we chose to use *U. maydis* growth data from co-culture, as opposed to growth alone, because the higher sample size yields a more reliable estimate of error. It is impossible to measure virulence in the plant and growth *in vitro* simultaneously for a single fungal culture, thus we obtained paired data points for *U. maydis* dikaryon genotype growth *in vitro* and virulence toward the

plant. We ran a correlation between mean strain growth and mean strain virulence for the 10 strains for which we had both measurements. To account for within strain variance, we used a parametric bootstrap to generate simulated data sets of strain growth *in vitro* and strain virulence *in plantae.* Strain means and variances used in the bootstrap were obtained from a linear model with the *gendata* function from the package of the same name. Regression slopes for the simulated data sets were calculated using the lm function. Sample regression slopes were recorded and tested for difference from zero under 1000 resampled datasets.

Results

We conducted two types of experiments. First, to assess genetic variation for *U. maydis* virulence towards the maize host, we inoculated maize plants with differing *U. maydis* dikaryon genotypes in greenhouse conditions. Plant size at the end of the experiment was used as a measure of virulence, or harm to the host. Second, to assess genetic variation for interactions between symbiotic microbes that commonly occur in maize, we measured and compared colony growth rates *in vitro,* in co-culture and culturing strains of each species alone. In these experiments, we used BOA to determine effects of a plant defensive compound on the outcomes of fungal interactions *in vitro.* Lastly, we examined evidence for potential trade-offs between growth rates in vitro and virulence for *U. maydis.*

Variation in U. maydis virulence towards the maize host

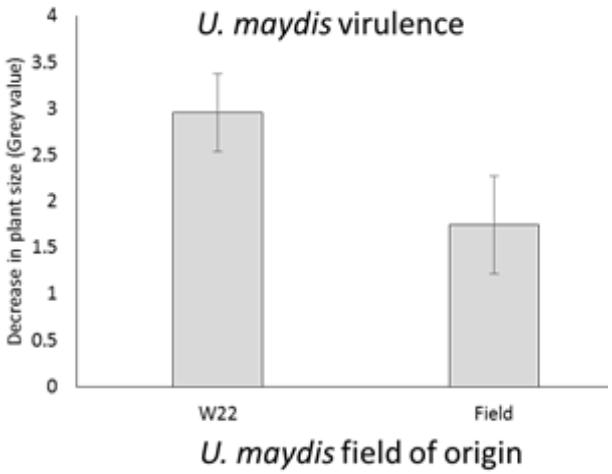

Figure 3.1: Population level variation in *U. maydis* virulence. Virulence is evaluated as the decrease in plant size due to infection with *U. maydis* relative to control plants not inoculated with *U. maydis*. *U. maydis* strains from the W22 field were significantly more virulent than strains from field corn ($p< 10^{-8}$). Error bars show 95% confidence intervals calculated from the pooled variance of the difference between infected and control plants.

The results of the two factor ANOVA (Table 3.3) showed that plants inoculated with *U. maydis* showed significantly less growth than did control plants, which were inoculated with sterile water. Additionally, mean virulence for strains isolated from the W22 field was significantly ($p<10^{-8}$) greater than virulence for strains from the field corn site (Figure 3.1). To determine whether co-occurrence of the two fungal species might cause selection for greater *U. maydis* virulence, we asked whether co-infection status predicted *U. maydis* virulence. Results showed no significant difference in virulence due to co-infection status at isolation (p=0.77). *U. maydis* strains that were isolated from the

same plant tissue as *F. verticillioides* showed similar virulence to strains that were isolated from tissues without *F. verticillioides*.

Fungal interactions in vitro - U. maydis

We cultured *U. maydis* and *F. verticillioides* strains *in vitro* to determine if *F. verticillioides* strains varied in their ability to inhibit *U. maydis* growth. In the co-inoculation experiment, 62 plates were unmeasurable either due to bacterial contamination or colony shape that was difficult to measure. These missing data was replaced with the grand mean of the co-inoculation experiment, generating a conservative estimate of statistical significance.

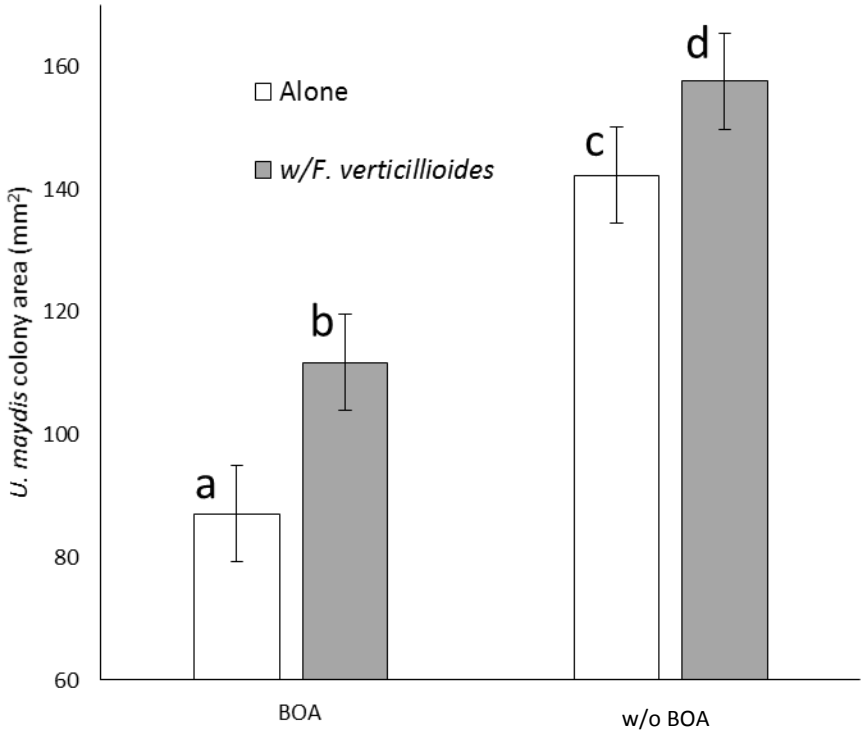

Figure 3.2: Mean *U. maydis* strain growth after five days, alone and in co-culture with *F. verticillioides*, in the presence or absence of BOA. White bars show *U. maydis* colony growth when cultured alone. Grey bars show mean *U. maydis* colony growth when co-cultured with *F. verticillioides*. Error bars show standard errors. Lowercase letters above each bar show significant differences as determined by a post-hoc Tukey's HSD test. These data show that mean *U. maydis* strain growth was greater in the presence of *F. verticillioides* than when *U. maydis* was grown alone. BOA restricts the growth of *U. maydis* although proportionally less so in the presence of *F. verticillioides*

U. maydis colony growth alone and in co-culture with F. verticillioides

We evaluate the effect of three factors on *U. maydis* colony growth using a linear mixed model; the defense compound BOA, field of origin, and the co-infection status of *U. maydis* at isolation. The defense compound BOA resulted in significantly smaller *U. maydis* colonies across all treatments ($p<<0.05$, Figure 3.2 white bars). In contrast, no significant effect of either field of origin ($p=0.122$; Field corn or W22) or of co-infection status ($p=0.770$) was detected for *U. maydis* growth in culture. In addition, we failed to find a correlation between *U. maydis* colony size and *F. verticillioides* colony size ($p=0.92$).

Contrary to expectations that *F. verticillioides* would inhibit *U. maydis* growth *in vitro*, we found that *U. maydis* grew faster in the presence of *F. verticillioides* ($p<10^{-5}$)

than when cultured alone. The linear mixed model analysis found no significant difference in growth of *U. maydis* due to the co-infection status of co-cultured *F. verticillioides* strains (see Table 3.4) suggesting that if co-occurrence of these two fungi causes selection for growth in competition, it is occurring on a population level rather than at the individual plant level. Results suggest that when co-cultured with *F. verticillioides,* the mean growth of *U. maydis* strains from field corn was greater than the mean growth of strains from the W22 field, however, the results were not strongly significant (p=0.052).

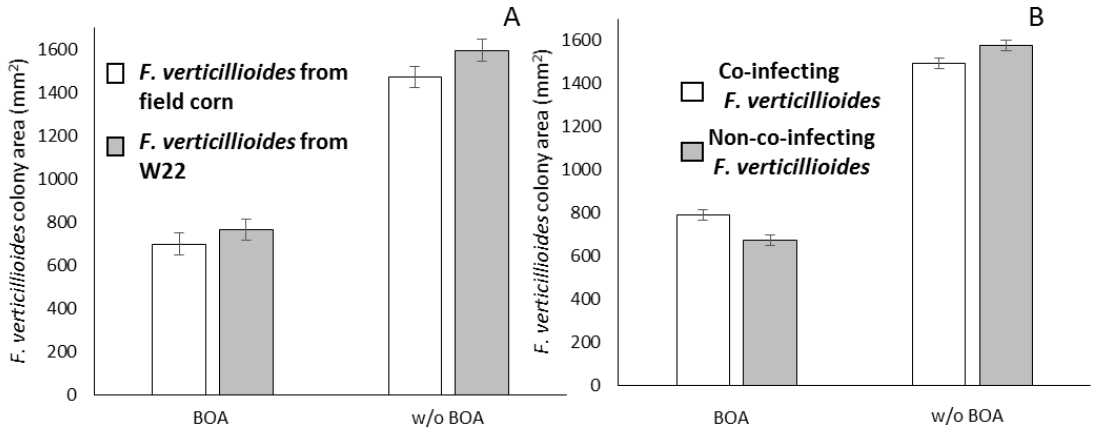

Figure 3.3: *F. verticillioides* growth *in vitro*. In each panel, *F. verticillioides* colony growth in PDA media with 1% BOA (left columns) and PDA without BOA (right columns) is shown. Panel A shows the effect of *F. verticillioides* field of origin on growth. Panel B shows the effect of *F. verticillioides* co-infection status on colony growth. Error bars show the standard error of each mean.

F. verticillioides growth in vitro, alone and in co-culture

We measured growth *in vitro* of the endophyte of maize, *F. verticillioides*, to determine if *F. verticillioides* growth varied between field, co-infection status, and with their response to the plant defense compound BOA. As expected, BOA significantly inhibited *F. verticillioides* growth ($p<<0.05$). We saw no significant difference in *F. verticillioides* growth response to BOA due to the field of origin as a main effect ($p=0.784$). We also failed to detect a difference in growth of *F. verticillioides* strains due to their status as co-infecting and not co-infecting with *U. maydis* ($p=0.133$, see Table 3.5). *U. maydis* had little effect on *F. verticillioides* growth and this was true regardless of the field of origin for the *U. maydis* strains ($p=0.8$). Likewise, there was no significant difference between *F. verticillioides* growth when partnered with *U. maydis* strains either co-infecting or not co-infecting the same tissue at isolation ($p=0.95$).

We tested for second order effects between *F. verticillioides* growth and the plant defense compound BOA to determine if the response of *F. verticillioides* strains to the plant defense compound depended on either co-infection status or field of origin. We found three significant second order effects. The mixed model showed significant interactions in *F. verticillioides* growth between strain co-infection status and the defense compound BOA ($p<10^{-9}$). Additionally, we detected a significant interaction between *F. verticillioides* field of origin and the presence of BOA in the media ($p<0.03$) and significant interactions between BOA and the field of origin of the co-cultured *U. maydis* strain. A post hoc was performed using a Tukey HSD on linear mixed model for *F. verticillioides* growth. The post hoc analysis showed significant differences between growth and without BOA growth, as expected, but failed to detect significant differences

between *F. verticillioides* field of origin or co-infection status. That the post hoc Tukey HSD failed to find significant interactions that were detected by the linear mixed model may be because post hoc pairwise comparisons tend to be underpowered (Ruxton and Beauchamp 2008). We therefore focus on differences in means between treatment combinations within significant interactions affecting *F. verticillioides* growth as shown by the linear mixed model approach.

F. verticillioides strains from the two fields grew at similar rates when plated with BOA, however strains from the W22 field grew faster than the field corn strains when in media without BOA (Figure 3.3A) suggesting that while strains from the W22 field grow faster, they are more sensitive to BOA. We also found a significant ($p<<0.05$) interaction of effects of *F. verticillioides* co-infecting status and the presence of BOA in the media (Figure 3.3B). When grown in media containing BOA, *F. verticillioides* strains co-infecting the same plant tissue as *U. maydis*, grew faster than strains that were isolated from plant tissues without *U. maydis*. When grown in media without BOA we found the opposite, co-infecting strains grew slower than strains that were not co-infecting with *U. maydis*.

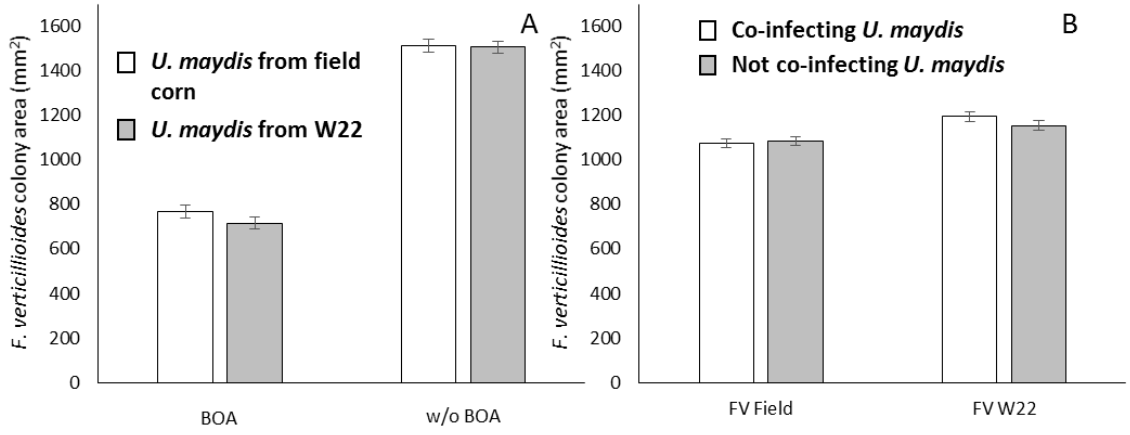

Figure 3.4: Effect of *U. maydis* on mean *F. verticillioides* growth *in vitro*. Panel A shows the effect of *U. maydis* strains from field corn (white bars) and W22 (grey bars) on the mean growth of *F. verticillioides.* Mean growth of colonies grown in media with BOA are shown in the left columns and media without BOA in the right columns. Panel B shows the effect of the *U. maydis* strain co-infection status on *F. verticillioides* growth. White bars show the mean growth of *F. verticillioides* strains from field corn and grey bars show mean growth of *F. verticillioides* strains from the W22 field. Error bars show the standard error of each mean.

U. maydis' effect on *F. verticillioides* growth was also dependent on the presence of BOA (Figure 3.4A). When grown in media containing BOA, *U. maydis* strains from the W22 field led to lower growth in the partnered *F. verticillioides* colonies than did *U. maydis* strains from field corn. However, there was no difference in the effect of *U. maydis* strains from different fields on *F. verticillioides* when grown without BOA. These results suggest that the *U. maydis* strains from the W22 field inhibit *F. verticillioides*

growth more on average than do *U. maydis* from field corn, but that difference is only detectible in the presence of BOA.

Finally, we saw a significant (p=0.035) interaction term for *F. verticillioides* field of origin and the co-infection status of the *U. maydis* strain on *F. verticillioides* growth in co-culture (Figure 3.4B). *F. verticillioides* strains from the W22 field grew faster when co-cultured with *U. maydis* strains that were isolated from the same tissue as *F. verticillioides* (co-infecting). However, important to note that the effect size was very small and that this is a potentially spurious result.

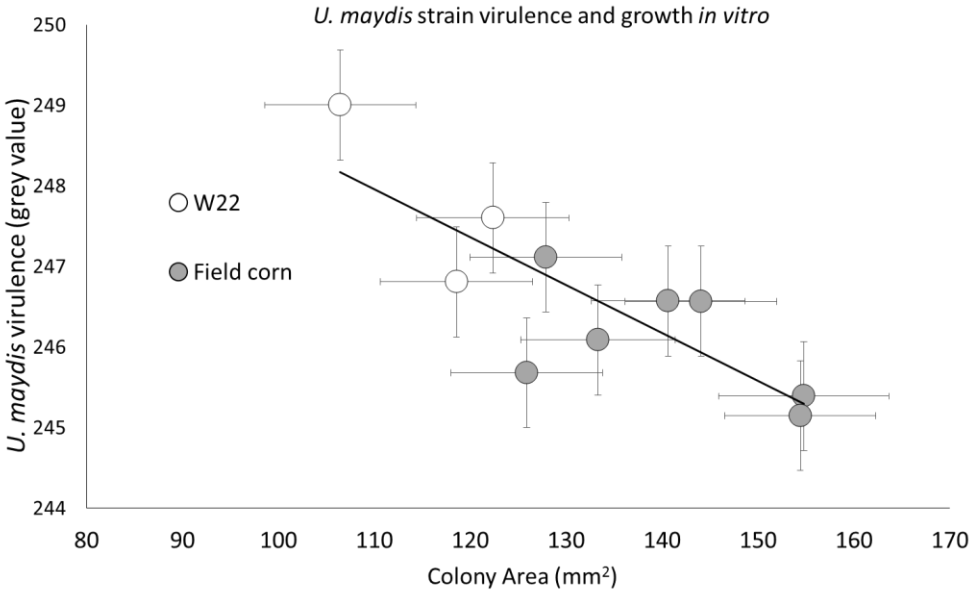

Figure 3.5: Correlation between *U. maydis* growth during *in vitro* co-culture with *F. verticillioides,* and the virulence of the same strains in maize. Open markers indicate

results for strains from the W22 field and grey markers indicate results for strains from field corn. Error bars indicate 95% confidence intervals for each strain using the variance in growth and virulence for each strain. Analysis of the data generated by the parametric bootstrap showed that the regression slope differed significantly from zero (p<0.036) with a slope of -6.7 and an R^2 of 0.4976.

We compared the growth *in vitro* virulence *in vivo* of the same dikaryon genotypes to determine if tradeoffs between saprotrophic and virulence traits might constrain the evolution of virulence. Results demonstrate negative correlation between strain virulence and strain growth in competition (Figure 3.5) suggesting negative pleiotropy between these traits. Thus, selection on virulence may be constrained by selection on saprotrophic traits, if growth rate is important to survival of *U. maydis* in the soil.

Discussion

This study assessed variation in a pathogen's virulence and in an endophyte's inhibition of that pathogen. We asked if populations of the pathogen *U. maydis* exhibited genetic variation in virulence, the degree of harm to the host. We also asked if populations of the fungal endophyte *F. verticillioides* showed genetic variation in the degree to which they inhibit *U. maydis* growth in culture, as a proxy for a host defensive trait. We found that strains of the pathogen *U. maydis* from a field corn population exhibited lower virulence than strains from the W22 field. Contrary to our expectations, we found that *U. maydis* growth *in vitro* increased in the presence of *F. verticillioides*,

which we had assumed acted as a defensive mutualist. *U. maydis* increased growth in the presence of *F. verticillioides* suggests that *U. maydis* facultatively increases its growth rate when it detects *F. verticillioides*. Finally, we detected a negative correlation between *U. maydis* growth *in vitro* and its virulence in the plant suggesting a tradeoff between saprophytic and parasitic life history traits.

Pathogen virulence

We found *U. maydis* strains isolated from the W22 field exhibited greater mean virulence than strains isolated from field corn. Given that just one field of each type of maize was sampled to obtain these strains, we are unable to attribute differences between the pathogen populations to differences in the host population. Two important ecological factors that influence virulence evolution are multiple infections within hosts and susceptible host density (Ebert and Mangin 1997). Under multiple infections, if one parasite kills its host all co-infecting parasites die as well. Therefore, selection favors virulent parasites that can transmit to new hosts before another parasite kills the current host (Ebert and Mangin 1997). However, as plants from both fields were colonized by endophytic fungi and endophytic fungi are relatively benign in their effects on the host, co-infection with endophytes per se is unlikely to explain the difference in virulence between populations. Further studies would be needed to determine if other symbiotic species colonizing W22 are more virulent than symbiotic species colonizing field corn. We look to the second possibility, that transmission success rate may be affecting virulence evolution.

A wide body of theory deals with role of gene for gene interactions in the evolution of host pathogen systems (see review by Hulbert et al. 2001). In a gene for gene system, hosts can evolve "resistance genes" which prevent infection. Conversely, pathogens can evolve "virulence genes" which allow a pathogen to infect resistant hosts. In gene for gene systems host resistance is widely expected to select for virulence genes to evade host resistance genes (McDonald and Linde 2002). However, the term virulence in gene for gene systems only refers to a pathogens ability to infect its host. Thus, theory regarding gene for gene evolution is silent on the evolution of virulence as used in this study, the degree to which a pathogen harms its host.

Transmission success is determined by host and parasite population genetic structure (de Wit 1992). Because field corn plants are more resistant to *U. maydis* infection than W22 plants, selection may be favoring low virulence strains that maintain longer, less damaging infections in field corn populations. That differences in *U. maydis*' ability to infect its hosts may alter selection on virulence is especially interesting in light of *U. maydis*' relationship with *F. verticillioides*. *F. verticillioides* has been shown to detoxify plant defense compounds (Richardson and Bacon 1995; Glenn et al. 2003) and might increase *U. maydis* infectivity. The potential for BOA to play a mediating role in interactions between *U. maydis* and *F. verticillioides* is of particular interest in light of our findings that *F. verticillioides* growth differed based on the field of origin and co-infection status, but only in the presence of BOA.

F. verticillioides growth in vitro

BOA affected *F. verticillioides* growth in two ways. First, *F. verticillioides* from the W22 field grew significantly faster than strains from field corn, but this difference was only significant in the absence of BOA. Thus, while the W22 *F. verticillioides* strains may grow faster than field corn strains, our results show that they are not as tolerant of BOA. Additionally, we found that *F. verticillioides* that was isolated from the same tissue as with *U. maydis* grew significantly faster than strains that were isolated from plants without *U. maydis*, but only in the presence of BOA. Because BOA detoxification can facilitate growth of other fungi (Saunders and Kohn 2009), it is possible that *F. verticillioides* strains with higher rates of BOA detoxification facilitate *U. maydis* infection and are therefore more likely to be found co-infecting with *U. maydis.* Our result that co-infecting *F. verticillioides* grew faster than not co-infecting strains in the presence of BOA and slower in its absence, suggests that detoxifying BOA provides an advantage when BOA present but comes at a cost when BOA is absent.

U. maydis growth in vitro

We measured fungal growth *in vitro* to look for genetic variation in the degree to which *F. verticillioides* inhibits *U. maydis* growth. It is important to note that this study only examined growth before the fungi came into direct contact. *F. verticillioides* has been shown to significantly reduce *U. maydis* biomass when *F. verticillioides* mycelia grow over *U. maydis* colonies *in vitro* (Rodriguez Estrada et al. 2011) and to reduce *U. maydis* growth in the plant (Rodriguez Estrada et al. 2012). However, counter to our expectations, co-culturing with *F. verticillioides* actually resulted in increased *U. maydis*

growth compared to growth alone, although the increase was only significant in the presence of the defense compound BOA. This increase in growth could either be due to *U. maydis* being stimulated to grow faster in the presence of *F. verticillioides,* or more likely, *F. verticillioides* detoxifying BOA (Saunders and Kohn 2008). The ability to detoxify BOA varies significantly among strains (Richardson and Bacon 1995; Glenn et al. 2003). One might expect then, that the amount of detoxification is proportional to the size of the *F. verticillioides* colony. However, we were unable to detect a correlation between *U. maydis* and *F. verticillioides* growth. Nonethess, given our demonstration of increased *U. maydis* growth in the presence of *F. verticillioides*, *F. verticillioides* may act as both an inhibitor and facilitator of *U. maydis*. While *F. verticillioides* inhibits *U. maydis* growth within the plant and reduces effects of disease (Lee et al. 2009), *F. verticillioides* may also help *U. maydis* evade plant defenses and facilitate infection.

Alternatively, *U. maydis* may be stimulated to grow faster due to the presence of an enemy. Fungi have been known to increase growth in the presence of metabolites from other species (Heilmann-Clausen and Boddy 2005). Unicellular organisms face an inherent tradeoff between growth rate and efficiency (Molenaar et al. 2009). When faced with a competitor, *U. maydis* may be switching to a less efficient but more rapid growth strategy. It has been shown that *U. maydis* facultatively expresses a variety of compounds in the presence of *F. verticillioides* and that *U. maydis* growth is significantly impaired after being overrun by *F. verticillioides* (Rodriguez Estrada et al. 2011). While for us humans, the idea that a microbe may want to "run away" from a competitor is attractive, *U. maydis* colonies grow undirected and thus at least half of the cells are growing toward

F. verticilloides. Because we measured colony growth before the fungi came into direct contact, *U. maydis* may be detecting a diffusible compound, or a change in nutrient status of the media caused by *F. verticillioides*.

Correlation between growth and virulence

We compared the growth of *U. maydis* strains *in vitro* with virulence *in vivo* to ask if the evolution of virulence might be constrained by a tradeoff between saprotrophic and symbiotic traits. If growth *in vitro* corresponds to virulence *in vivo* (Caraco and Wang 2007), we expect a positive correlation between strain growth *in vitro* and strain virulence. Contrary to expectations, results show that *U. maydis* strains that grow faster *in vitro* cause less damage to their hosts than slower growing strains. This results suggests support for a tradeoff between growth *in vitro* (saprophytic) and virulence. Many symbionts are subject to tradeoffs between traits affecting different life history stages (Woodhams et al. 2008; Morris et al. 2009). Rust fungi, for example, have been shown be subject to tradeoff between infectivity as determined by classic gene-for-gene virulence factors and a quantitative effect of an increasing number of virulence factors on the latent period between initial infection and sporulation (Bruns et al. 2012). Among symbionts with a free-living life history stage, it is sometimes assumed that high survival rates during the free-living stage allows for the evolution of greater virulence (Bonhoeffer et al. 1996; Gandon 1998). However, most studies have focused on parasites with non-replicative free-living forms (Caraco and Wang 2007) or opportunistic pathogens (Brown et al. 2012) that do not need a host to complete their life cycle. Less attention has been

paid to pathogens that can grow and replicate outside of the host. Because *U. maydis* can grow as a free living yeast but requires a host for sexual reproduction (Bölker 2001; Pérez-Martín 2006), this study provides insight into an overlooked life history strategy. The negative correlation between growth *in vitro* and virulence suggests that virulence in *U. maydis* may come at a double cost, first by potentially killing the host (Lenski and May 1995), and second by reducing growth in the saprophytic stage.

Conclusion

The goal of this study was to determine evidence for genetic variation in pathogen virulence and symbiont mediated host-defensive traits. We found significant differences in in the levels of virulence between two populations of the smut fungus *U. maydis*. Additionally, our results show that virulence to the plant is correlated with slower growth rates *in vitro.* Therefore, selection on virulence may be a function of the ecology and community composition during a symbiont's free living stage, as well as the conditions within the host. Furthermore, we show that interactions between organisms may change significantly depending on the environmental conditions under which the interactions occur. These data show the importance of examining a symbiont's entire life history, as well as its host and symbiotic community context, when attempting to elucidate the evolutionary and ecological forces that shape its relationship to its host.

Table 3.1: *F. verticillioides* strain designations

F. verticillioides strain ID	Isolated with *U. maydis*?	Field of origin
GF11	Yes	Field corn
GF110	Yes	Field corn
GF15	No	Field corn
GF2	Yes	Field corn
GF40	No	Field corn
GF46	No	Field corn
GF49	No	Field corn
GF80	Yes	Field corn
GSP105	Yes	W22
GSP125	No	W22
GSP134	Yes	W22
GSP194	No	W22
GSP24	Yes	W22
GSP260	No	W22
GSP345	No	W22
GSP50	Yes	W22

Table 3.2: *U. maydis* strain designations

U. maydis strain ID	Isolated with *F. verticillioides*?	Field of origin	*In vitro*	*In vivo*
IV-F-11-13E	No	Field corn	X	X
IV-F-1-21E	Yes	Field corn	X	X
IV-F-13-6W	Unknown	Field corn		X
IV-F-16-15E	No	Field corn	X	X
IV-F-1-7W	Yes	Field corn	X	X
IV-F-18-13W	Yes	Field corn		X
IV-F-3-3W	No	Field corn	X	X
IV-F-6-14W	Unknown	Field corn		X
IV-F-6-3E	No	Field corn	X	X
IV-F-6-3W	Yes	Field corn	X	
IV-F-7-13W	Yes	Field corn		X
IV-F-9-10E	Yes	Field corn	X	X
V-SP-W22-1	No	W22	X	X
V-SP-W22-13	Yes	W22	X	
V-SP-W22-17	Yes	W22	X	
V-SP-W22-18	No	W22	X	
V-SP-W22-25	No	W22	X	
V-SP-W22-3	No	W22		X
V-SP-W22-31	No	W22		X
V-SP-W22-32	Yes	W22		X
V-SP-W22-33	Yes	W22		X
V-SP-W22-34	No	W22		X
V-SP-W22-35	No	W22		X
V-SP-W22-37	No	W22		X

V-SP-W22-39	No	W22	X	
V-SP-W22-4	Yes	W22	X	X
V-SP-W22-5	Unknown	W22		X
V-SP-W22-6	No	W22		X
V-SP-W22-7	Yes	W22	X	X
V-SP-W22-8	No	W22		X

Table 3.3: Results of ANOVA analysis of *U. maydis* virulence *in vivo*. This table shows the effect of two qualities of each strain, the strain field of origin (field), and co-infection status at isolation (co-infect) on *U. maydis* growth. This analysis shows that *U. maydis* strains from the two fields differed significantly in virulence toward maize.

	d.f.	SumSq	MeanSq	F	p value
Field	1	207	207.33	29.705	7.51E-08*
Co-infect	1	1	0.61	0.088	0.767
Residuals	567	3957	6.98		

* significance $p < 0.05$

Table 3.4: Results of linear mixed model analysis of *U. maydis* growth *in vitro*. We tested for variation in growth of *U. maydis* strains and the effect of partnered *F. verticillioides* colonies (FV) on *U. maydis* growth. This table shows the effects of the field of origin (field), and co-infection status at isolation (coinfect) on variation in *U. maydis* growth. Additionally, we tested the effect of the plant defense compound BOA and the size of the co-cultured *F. verticillioides* colony (partner area) on *U. maydis* growth *in vitro*.

	U. maydis colony size			
	Estimate	Std. Error	t value	p value
BOA	0.25	0.12	2.11	0.035 *
FV field	-0.14	0.07	-1.94	0.052
UM field	-0.13	0.09	-1.55	0.122
FV coinfect	0.01	0.07	0.18	0.859
UM coinfect	-0.03	0.09	-0.29	0.770
Partner area	0.00	0.00	-0.10	0.923
BOA:FV field	0.10	0.07	1.51	0.130
BOA:UM field	-0.05	0.06	-0.81	0.419
BOA:FV coinfect	-0.02	0.07	-0.30	0.764
BOA:UM coinfect	0.07	0.06	1.03	0.303
BOA:Partner area	0.00	0.00	0.23	0.821
FV field:UM field	-0.02	0.03	-0.60	0.549
FV field:FV coinfect	0.03	0.04	0.81	0.420
FV field:UM coinfect	0.00	0.03	-0.07	0.941
FV field:Partner area	0.00	0.00	0.11	0.912
UM field:FV coinfect	-0.01	0.03	-0.47	0.641
UM field:UM coinfect	0.10	0.09	1.17	0.240
UM field:Partner area	0.00	0.00	0.61	0.544
FV coinfect:UM coinfect	-0.04	0.03	-1.39	0.165
FV coinfect:Partner area	0.00	0.00	0.46	0.646
UM coinfect:Partner area	0.00	0.00	0.20	0.844

* significance $p < 0.05$

Table 3.5: Results of linear mixed model analysis of *F. verticillioides* growth *in vitro*. We tested for variation in growth of *F. verticillioides* strains due to effects of partnered *U. maydis* colonies on *F. verticillioides* (FV) growth. This table shows the effect of two qualities of each strain, the strain field of origin (field), and co-infection status at isolation (coinfect) on *F. verticillioides* growth. Additionally, we tested the effects of the plant defense compound BOA and the size of the co-cultured *U. maydis* colony (partner area) on *F. verticillioides* growth *in vitro*.

F. verticillioides colony size

	Estimate	Std. Error	t value	p value	
BOA	599.37	54.49	11.00	0.000	*
FV field	27.64	100.93	0.27	0.784	
UM field	12.53	49.47	0.25	0.800	
FV coinfect	-134.27	89.38	-1.50	0.133	
UM coinfect	3.25	51.85	0.06	0.950	
Partner area	0.58	0.38	1.53	0.127	
BOA:FV field	59.73	27.74	2.15	0.031	*
BOA:UM field	69.84	27.76	2.52	0.012	*
BOA:FV coinfect	168.74	27.16	6.21	0.000	*
BOA:UM coinfect	40.08	27.47	1.46	0.145	
BOA:Partner area	-0.13	0.32	-0.41	0.683	
FV field:UM field	-11.73	23.47	-0.50	0.617	
FV field:FV coinfect	107.64	124.64	0.86	0.388	
FV field:UM coinfect	-49.25	23.40	-2.10	0.035	*
FV field:Partner area	0.07	0.32	0.22	0.826	
UM field:FV coinfect	-36.93	23.30	-1.58	0.113	
UM field:UM coinfect	29.74	39.93	0.74	0.456	
UM field:Partner area	-0.47	0.32	-1.45	0.147	
FV coinfect:UM coinfect	4.93	23.28	0.21	0.832	
FV coinfect:Partner area	0.31	0.32	0.99	0.323	
UM coinfect:Partner area	-0.22	0.32	-0.68	0.498	

* significance $p < 0.05$

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