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DEFENSIVE SYMBIOSIS

Defensive mutualisms: do microbial interactions within hosts drive the evolution of defensive traits?

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Summary

1. We examine theoretical and empirical results to determine the importance of microbe–microbe interactions in the evolution of defensive traits.

2. Theoretical models show that the evolution of parasitism and the maintenance of mutualisms in multispecies interactions will depend on interactions with the host as well as with the defensive symbionts.

3. At the community level, selection for defensive traits will vary greatly with ecological context and such spatial or temporal variation itself may stabilize defensive mutualisms.

4. Studies of fungal endophytes within plant hosts demonstrate that endophytes acting as defensive mutualists may derive fitness benefits from the parasite as well as the host and suggest that interactions between co-occurring symbionts within hosts may lead to the evolution of virulence.

Key-words: antagonism, cheater, defensive mutualist, host, microbiome, mutualism, parasite, symbiosis, virulence

Introduction

Defensive symbionts pose particularly knotty but interesting problems for understanding the co-evolution of eukaryotic hosts and their symbiotic partners. While protection from parasites, enemies, or stressful abiotic conditions is of clear benefit to the host, the paradigm of a defensive symbiont as a mutualist conferring uniform benefit to the host is giving way to models of variable, context-dependent costs and benefits (Jaenike *et al.* 2010; Saunders, Glenn & Kohn 2010; Fenton *et al.* 2011; Rudgers *et al.* 2012). The particular focus of this study is to understand the impacts of the many and diverse microbial symbionts of eukaryotic hosts, the microbiome, on the evolution of defensive traits. We primarily consider adaptive evolutionary processes, and thus will focus on the ecological factors that generate selection on genetic variation within species for traits that affect interactions among species. Few species occupying a microbiome have universally negative or positive interactions with their host and the myriad co-occurring symbionts, thus we are necessarily concerned with forces that push symbiont populations along a continuum between parasitism and mutualism (Fellous & Salvaudon 2009).

We forward the thesis that 'the interactions among microbes within hosts' may often generate as strong selection for defensive traits 'as do interactions of the host with its parasites or enemies'. To the extent that such microbe–microbe interactions provide selection on traits of organisms that we also consider defensive symbionts, protection of the host could be a by-product, rather than the primary source of selection on the function of defensive traits. Some defensive traits may have evolved more to protect the microbe than to protect the host. Of course, the host cannot be ignored, and our task becomes identifying the important factors affecting fitness outcomes for each of the interacting species: host, parasite and members of the host's microbiome that include defensive symbionts. A subtext of the study is that the implicit assumption of endosymbionts as having inherently mutualistic or parasitic fitness effects on their hosts limits our study and hampers our understanding of their evolution. Consequently, we want to use terms that allow for variable nutritional modes and outcomes (Box 1). We use symbiosis as originally defined by de Bary (1879) to describe the entire spectrum of interactions between two closely associated

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organisms without assumption of positive or negative fitness outcomes. We use mutualism to describe a net beneficial interaction between organisms and parasitism to describe interactions between organisms that result in a net cost to one. Following the entomological and ecological literature, and because parasites typically connote infection and damage to the host from within, we also use the term enemy to describe organisms such as herbivores that often attack a host from the outside.

Box 1: Definitions of terms

We use 'symbiosis' as defined originally by Anton de Bary (1879) and in the broad sense of any close ecological or evolutionary interaction of two or more distinct organisms

Endophyte	Microbial organisms living within plants without causing apparent disease symptoms (Wilson 1995). In this study, we are not assuming the symbiotic nutritional mode of endophytes as parasite, mutualist or commensal
Mutualist	A symbiotic organism that confers net fitness benefits to the host and that gains a net benefit from the host. Defensive mutualists decrease negative impacts of parasites or enemies on hosts by a variety of mechanisms and are thus seen to 'defend' their hosts
Parasite	A symbiotic organism that confers net fitness costs to the host and that gains a net fitness benefit from the host. In this study, we include cheaters (e.g. Kiers *et al.* 2010) and exploiters (e.g. Jones, Ferriere & Bronstein 2009) as parasites
Enemy	Any organism that consumes or damages tissues of the host, often from the outside. For example, we include herbivores (e.g. Chesson 2000) as enemies of a plant host

In this study, we focus on fungal endophytes of plant hosts to understand the evolution of multispecies microbial systems that include defensive symbionts and seek common themes in other multispecies interactions. The grass endophyte Epichloë has long provided a quintessential plant system and yielded key insights into the underlying genetic mechanisms and ecological outcomes for interactions between plant host, defensive symbiont and herbivores (Clay 1988, 1999; Schardl, Leuchtmann & Spiering 2004). Nonetheless, the vertically transmitted Epichloë represents just the tip of the iceberg for fungal diversity within plants (Carroll 1988; Rodriguez *et al.* 2009). When we include the horizontally transmitted fungal endophytes, those fungi that occur within plants without causing apparent disease symptoms (Wilson 1995), it is clear that not all endophytes may benefit the host (Carroll 1988) and that transitions to parasitism are not uncommon on a phylogenetic scale (Arnold *et al.* 2009). While the descriptions of endophytic organismal diversity (Arnold 2007; U'Ren *et al.* 2012) and of interactions between endophytes, plant hosts and enemies are increasingly detailed (Richmond *et al.* 2004; Saunders & Kohn 2009; Rodriguez Estrada *et al.* 2012), two broad problems need attention. First, a stronger theoretical framework that recognizes the ecological context-dependence of symbiotic evolution will help frame testable hypotheses. That context might encompass the identity and genetic relationship of interacting symbionts (e.g. Gardner, West & Buckling 2004; Vigneux *et al.* 2008), host identity and genotype (e.g. Pan & May 2009), or the specific mechanisms of interaction among the assembled community members (Buckling & Brockhurst 2008; Saunders, Glenn & Kohn 2010). Secondly, empirical measurements that untangle genetic and environmental sources of variation in the fitness costs and benefits of symbioses will provide insight into important evolutionary forces and inform model parameters. Both these challenges have at their centre the inherent ecological instability and consequent variation in individual fitness effects within multispecies assemblies (Rigaud, Perrot-Minnot & Brown 2010).

WHAT WE UNDERSTAND FROM THEORY

Considering the evolutionary or ecological stability of defensive symbioses immediately poses a conceptual conundrum. Ecological theory suggests that mutualists and parasites will more likely coexist when the strength of intraspecific competition is greater than interspecific competition (Chesson 2000). Yet, evolutionary theory suggests that competition among multiple genotype infections will often drive the evolution of greater virulence towards the host of one infecting species or population (Alizon *et al.* 2009; Weyl *et al.* 2010; Lopez-Villavicencio *et al.* 2011), assuming that genetic variation for traits affecting these interactions exists. Consequently, we arrive at a question often posed for mutualisms more broadly: how do we explain the evolutionary stability of defensive mutualists, those symbionts that defend their hosts against parasites or enemies and result in a net fitness benefit to the host? Given the diversity of organisms in defensive mutualisms, there are likely many-fold specific answers. We concentrate on evaluating the relative importance of two main sources of constraint on the evolution of a parasite's increased damage to its host (virulence): constraints exerted by the host, such as sanctions or partner choice (e.g. Weyl *et al.* 2010), and constraints exerted by the interacting microbial symbionts within the host (Duffy, Schouten & Raaijmakers 2003; Gardner, West & Buckling 2004).

Examining the effects interactions of differing genotypes within species, theory predicts that competition among multiple infecting genotypes will select for greater virulence towards the host as a competitive mechanism to gain host resources (Nowak & May 1994; Alizon *et al.* 2009). As Ebert & Bull (2003) have noted, that prediction relies on positive association of competition and virulence traits. For example, a toxin that limits growth of competitors might also be a virulence factor damaging the host (Desjardins *et al.* 2005; Glenn *et al.* 2008). While formally, cooperation in defending the host could also evolve among closely related genotypes occupying common patches of host tissue (Axelrod & Hamilton 1981; West *et al.* 2002), for horizontally transmitted endophytes, it is unlikely that two closely related genotypes will arrive together on the same patch frequently enough for kin selection to be effective. The problem of understanding the evolutionary stability of defensive mutualists against competitive pressures from other symbionts becomes a task of understanding mechanisms that limit a 'cheater', a defensive mutualist that does not provide the host with services or goods but gains host resources as if it did. By our definition, such a 'cheater' is a parasite and the cost to the host is virulence. Assuming defence is costly, selection pressure maintaining defence must be the result of negative fitness outcomes for the host, for co-occurring microbial symbionts, or both.

Focusing first on host constraints, theory predicts the decay of mutualism unless the host sanctions limit cheater fitness. Sanctions can take the form of the host limiting resources supplied to cheater parasites (West *et al.* 2002; Kiers *et al.* 2010) or limiting the interaction duration (Johnstone & Bshary 2008) with these parasites in some way. Host sanctions can be seen in a resource perspective; a mutualism such as a mycorrhizal fungus that evolves optimal resource trading with its host may play 'keep away' and prevent cheater's access to nutrients (Bever *et al.* 2009; de Mazancourt & Schwartz 2010). Faced with a mixed population of mutualists and cheaters where sanctions are ineffective, a host may recognize and limit unproductive interactions with parasites before infection, exercising partner choice (Weyl *et al.* 2010), or screen out unproductive symbionts after infection (Archetti *et al.* 2011). However, while sanctions or partner choice models help explain the stability of one-to-one symbioses such as plants with nitrogen-fixing bacteria (Kiers *et al.* 2010) or systemic fungi such as Epichloë (Saikkonen *et al.* 1998), it is difficult to imagine effective host limits on non-beneficial endophytic symbionts if beneficial and parasitic genotypes occur within the same small patch of host tissue (Sachs & Simms 2006). The host may simply not be able to discriminate between symbionts and limits nutrient supply to the parasite over such a small scale, although this certainly bears further investigation. If so, the strongest selection would then fall on microbe–microbe interactions to limit the growth of parasitic cheaters. In these cases, outcomes should strongly depend on the mechanisms of interaction (Buckling & Brockhurst 2008). If the defensive mutualist has the ability to protect itself against its own toxins, interference with its con-specific competitors may engender a strong antagonistic response and an 'arms race' between these two microbial populations. Strong antagonism among symbionts may then limit the evolution of virulence (Koskella, Giraud & Hood 2006; Buckling & Brockhurst 2008) as long as the host is insensitive to the toxins. We point out, however, that many mechanisms of microbe–microbe interaction depend on first detecting differences between genotypes. We predict strong selection on the ability of the defensive symbiont to detect a parasite which could, in turn, engender rapid evolution of self/nonself recognition factors (e.g. Glass & Kaneko 2003). The evolution of attack traits such as cell wall-degrading enzymes (Karlsson & Stenlid 2008) or toxins (Jonkers *et al.* 2012) by the defensive symbiont should follow detection.

Clearly, rapid evolution has to be sourced from genetic variation within species but many defensive symbioses involve interactions among distantly related species. We then seek ecological processes that explain maintenance of host, enemy and defensive symbiont populations. Ecological and evolutionary models point towards coexistence of parasites and mutualists when intraspecific competition is greater than interspecific competition (Chesson 2000; Bronstein, Wilson & Morris 2003). Such systems may only be dynamically stable, however. For example, and relevant to our focus on endophytes, Lively *et al.* (2005) show that increased host fitness and population density resulting from the benefits of a vertically transmitted endophyte such as Epichloë can indirectly provide selection for increased virulence in a horizontally transmitted microbe such as a co-occurring endophyte. Similarly, Neuhauser & Fargione (2004) find that factors increasing host carrying capacity favour a shift of mutualism to parasitism. The seemingly counter-intuitive implication that a successful defensive mutualist may create conditions for the invasion of a parasite warrants empirical investigation.

If defensive mutualists increase availability of hosts, and thus indirectly create the conditions for enemy or parasite invasion, are these systems stable or is the extinction of one or more species' populations likely? Models predicting global extinction in a mass action environment (all experience each other's effects) can yield coexistence when temporal and spatial heterogeneity is taken into account (Prado & Kerr 2008; Nahum, Harding & Kerr 2011). For example, if spatially structured populations lead to more interactions among cooperators than with antagonists, cooperation can evolve even if the cost is high (Fletcher & Doebeli 2009). Translating the work of Ferriére, Gauduchon & Bronstein (2007) and Jones, Ferriére & Bronstein (2009) on pollinator systems to our questions about defensive mutualists, a kamikaze exploiter (parasite by our definition) may invade and crash high-resource host–mutualist interactions because the mutualist cannot out-compete the aggressive parasite. Such instability contrasts with the stability predicted in the models of de Mazancourt & Schwartz (2010) above because, in the case of the kamikaze exploiter, the host's interactions with the mutualist increases total host resources, increasing the opportunity for an invading parasite. Nonetheless, the three species may coexist if the parasite arrives within a zone of coexistence, that is, before the mutualist costs the host too much.

As the occurrence of multiple symbionts of varying effects on the host is the rule more than the exception (Sachs & Simms 2006; Oliver & Russell, this volume), we may reframe the question again: under what conditions is the evolutionary maintenance of a defensive mutualism more likely? If the level of defence against enemies and the benefit to the host are conditional on the local community of enemies, this could have a stabilizing effect on community composition (Rai, Freedman & Addicott 1983; Ringel, Hu & Anderson 1996; Gomulkiewicz, Nuismer & Thompson 2003). We can contrast our predictions for the evolution of defensive traits in systems of closely related organisms, with expectations in multispecies systems. Systems with defensive mutualists guarding a host against enemies are often dynamically stable, and such a varied community genetic context may produce selection for generalist strategies of defence and depend more on the evolutionary history of individual species. In cases of a constantly shifting community context, ecological phylogenetic approaches (e.g. Cavender-Bares *et al.* 2009) may be useful in identifying the evolutionary origin of constraints on parasites (Saunders, Glenn & Kohn 2010).

In summary, from theory, we can conclude that defensive mutualists are unlikely altruists and are left with the more difficult task of understanding the conditions under which a defensive mutualist should evolve and persist amid the complex microbial communities found in all hosts. In the ongoing evolutionary processes within species, the ability of a newly evolved parasite to invade depends not only on the strength and mechanisms of interactions between the parasite and host, but also on interactions with the ecological community within the host, the microbiome. At the level of interactions among more distantly related organisms, the stability of a defensive mutualist population may depend much more critically on the phylogenetic history of the particular species involved. Although it is not trivial to identify the most important partners in a microbiome, the task for the empiricist is to determine the availability of genetic variation for traits that affect interactions and fitness outcomes for individual species.

WHAT DO WE ACTUALLY OBSERVE?

Fungal endophytes provide excellent systems for the study of multiple symbiont interactions, work that will inform function for the endophyte fungi that demonstrate such a tremendous depth of diversity. Many fungal endophytes of plants belong to the ascomycetes, especially the Pezizomycotina, and occur in photosynthetic hosts as diverse as lichens, mosses and flowering plants (Arnold *et al.* 2009; U'Ren *et al.* 2012). Although the horizontally transmitted endophytic fungi defend their plant hosts against parasite infection (Arnold *et al.* 2003; Lee, Pan & May 2009) and serve as effective biocontrol agents (Backman & Sikora 2008), they are often closely related to pathogens, and the implicit assumption of endophytes as mutualists is probably not warranted (Saikkonen *et al.* 1998). Here, we use two case studies and several examples to support our position that microbe–microbe interactions within the host may push the evolution of a defensive symbiont towards mutualism or parasitism as strongly as do pairwise interactions of any of these symbionts directly with the host.

We have used the maize microbiome to address questions regarding the function of fungal endophyte communities because these communities are species diverse, some with apparent benefit to the host, many with no apparent effect at all and some closely related to maize pathogens (Kuldau & Yates 2000; Pan, Baumgarten & May 2008; Pan & May 2009; Saunders & Kohn 2009). Maize (*Zea mays* subspp. *mays*) is the domesticated descendent of teosinte and interestingly, as Native Americans traded it northward, they brought a host of symbionts with it, including *Ustilago maydis* (Munkacsi, Stoxen & May 2008). As in many plants, the endophytes of maize are largely ascomycetes (Kuldau & Yates 2000; Pan & May 2009) and grow as filamentous hyphae in the intercellular spaces between cells. In our work, we show that endophytic *Fusarium verticillioides* acts as a defensive mutualist as it slows the progress of disease caused by the pathogen, *U. maydis*. The evidence of a protective benefit is that plants infected with both *U. maydis* and *F. verticillioides* grow as large as control plants not infected by either fungus (Lee, Pan & May 2009; Rodriguez Estrada *et al.* 2012). Thus, in the presence of the corn pathogen, there is an apparent benefit to the host of harbouring *F. verticillioides* in vegetative tissues, and in the absence of the parasite, there is no apparent cost to plant growth of harbouring the endophyte because the growth of plants inoculated with *F. verticillioides* alone is the same as controls. Surprisingly, pathogen spore production is increased in the presence of *F. verticillioides*, apparently because the plants grow larger and are longer-lived than those infected with the pathogen alone. *Ustilago maydis* spore production is closely correlated with plant height (Lee 2010). The fitness outcomes in this three-way interaction are not predicted by summing expectations for two-way interactions, and so we ask, which partner pays the cost for protection against a host's enemies?

We addressed the underlying mechanisms yielding non-additive fitness outcomes using *in vitro* and *in planta* experiments (summarized in Fig. 1). Using *in vitro* experiments, we show that *F. verticillioides* produces several metabolites associated with slower growth of *U. maydis* (Rodriguez Estrada *et al.* 2011). Of these, fusaric acid cluster is up-regulated in response to the presence of *U. maydis* (Jonkers *et al.* 2012), in apparent agreement with *in planta* observations that *F. verticillioides* strains producing more fusaric acid more strongly limit the growth of *U. maydis* (Lee 2010). In the plant, quantitative PCR (qPCR) experiments demonstrated that *F. verticillioides* grows up to 10- to 15-fold more biomass in the presence of *U. maydis* than in the pathogen's absence, apparently gaining a growth benefit from the pathogen. The pathogen grows up to 30- to 40-fold less biomass in the presence of *F. verticillioides*, consistent with our observations of slower disease progress in the presence of the endophyte (Rodriguez Estrada *et al.* 2012). Moreover, *F. verticillioides* sometimes facilitates maize colonization by *U. maydis* (Lee, Pan & May 2009) and other maize-infecting fungi (Saunders & Kohn 2009) because it breaks down the BOA-related plant defensive compound (Glenn, Gold & Bacon 2002). Remarkably, the defensive symbiont

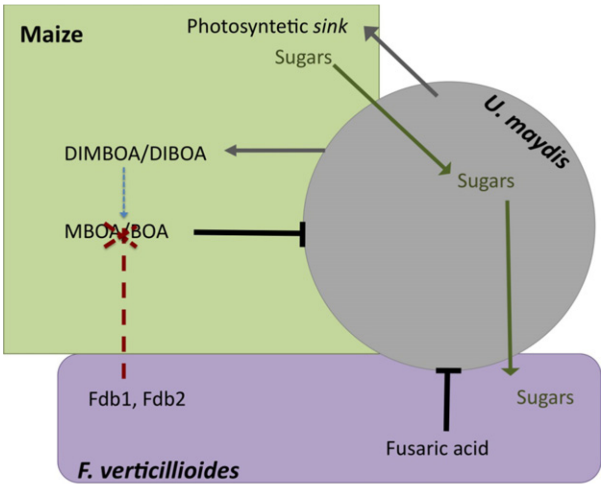

Fig. 1. A model for three-way interactions between maize, the pathogen *Ustilago maydis* and endophytic *Fusarium verticillioides* (adapted from Rodriguez Estrada *et al.* 2012). *Ustilago maydis* stimulates the production of maize antimicrobial BOA-related compounds which spontaneously degrade to MBOA and BOA (blue arrow). These inhibit *U. maydis* growth. *Fusarium verticillioides* produces two enzymes that degrade BOA compounds (Fdb1, Fdb2; Glenn, Gold & Bacon 2002) and may facilitate *U. maydis* entry in the plant. The endophyte also limits *U. maydis* growth by the production of secondary compounds such as fusaric acid. Infections of *U. maydis* tend to maintain maize leaves in a juvenile state longer (Doehlemann *et al.* 2008) suggesting that the pathogen extracts sugars subsequently acquired by *F. verticillioides*. Because the endophyte slows pathogen growth and disease symptoms, plants gain height growth comparable to that found in control plants without either fungus present. Thus, *F. verticillioides* is a defensive mutualist but may regulate the growth of the parasite to its own benefit, rather than that of the plant.

apparently modulates infection by the pathogen and gains a growth benefit from it.

Our working model is that the endophyte gains fixed carbon resources from the pathogen, and only indirectly from the plant (Fig. 1). *Ustilago maydis* maintains leaves in a juvenile state and as a carbon sink (Doehlemann *et al.* 2008) and thus serves as a conduit of sugars from the plant to the endophyte. Because *U. maydis* grows more slowly within the plant when the endophyte is present, but produces more spores over a longer infection period, pathogen fitness apparently increases in the presence of the endophyte. Such quantitative nonadditive interactions should stably maintain populations of plant, defensive mutualist, and pathogen as predicted by Jones, Ferriere & Bronstein (2009). In sum, we posit that the evolution of secondary metabolites, such as fusaric acid or fumonisins (Desjardins *et al.* 2005; Glenn *et al.* 2008), may be more strongly affected by fungal–fungal interactions than by direct selection on traits that serve to defend the host and increase host fitness. We are currently investigating the hypothesis that among-microbe interactions may drive either or lesser greater virulence in maize symbionts towards their host. In maize susceptible to both fungi, both fungi are often present and should strongly compete for host resources. Competitive interactions should select for greater virulence towards the host by at least one fungal partner. In contrast, in maize that is resistant to *U. maydis*, the pathogen does not easily access the host resources and its growth may be facilitated by the breakdown of plant defence compounds (as above) by the endophyte. To the extent that the endophyte facilitates the pathogen's growth in the host, this should provide selection for more cooperative than competitive interactions and dampen selection for greater virulence for either fungus.

Evidence that microbe–microbe interactions may drive the evolution of traits typically thought to function for defence of the host or its resources is found in other systems as well. For example, fungus-farming ants carry an actinomycete bacterium that provides antibiotic defence against *Escovopsis*, a parasite of the farmed fungus (Currie *et al.* 2003). To benefit the ant through protection of the resource, the antibiotic should show specificity and limit the growth of the parasite but not harm the farmed fungus. However, recent community level analyses by Sen *et al.* (2009) demonstrate several genotypes of the actinomycete bacterium on the ants, and their antibiotics do not apparently demonstrate the necessary specificity towards the parasite. While certainly the actinomycete behaves as a defensive mutualist (Cafaro *et al.* 2011), its presence may have some cost to the ant's resources if the antibiotic also limits the growth of the farmed fungus. Further, the antibiotics may have evolved in response to competitive interactions among actinomycete symbionts and have been implicated in defence against an entomopathogenic fungus (Mattoso, Moreira & Samuels 2012) as well. These observations support our thesis that the evolution of antibiotic diversity may result from competitive interactions among microbes co-occurring on a host, rather than from co-evolutionary arms races with presumed targets (Boomsma & Aanen 2009; Sen *et al.* 2009). Overall, observations from two very different systems, ant-microbe-farmed fungus, and the maize microbiome, suggest an evolutionary scenario of ongoing subterfuge to avoid recognition, cooperation, and defection, rather than out-and-out arms races between host and enemy.

Less attention has been paid to interactions among symbionts within a host where outcomes depend on the biotic and abiotic ecological context. For example, Vigneux *et al.* (2008) showed that the fitness outcomes of interactions between symbionts of a nematode parasite on an insect host depend on local population structure of symbionts and parasite. Here, bacterial symbionts that have co-evolved under conditions of high migration among subpopulations demonstrate greater levels of inhibition towards each other and lower virulence towards the nematode in which they reside, than do bacterial symbionts that have co-evolved in low-migration environments. Low-migration populations are apparently cooperating in attack of their nematode host which is then less virulent on the insect host. In another example, Jaenike *et al.* (2010) show that *Spiroplasma,* a bacterial symbiont of a Drosophila fly, is spreading in North America. *Spiroplasma* has little apparent impact on the occurrence of a *Wolbachia* symbiont in the same fly but does allow the fly to recover egg production in the presence of a nematode parasite. Because *Spiroplasma* benefits host reproduction in the presence of the nematode but has little effect in the absence of the parasite, it is a defensive mutualist. Interestingly for our thesis, some *Sprioplasma* interactions with the fly may be parasitic. Thus, a parasite may become a host's ally in many ways (Fellous & Salvaudon 2009). Bacterial or fungal symbionts which provide the host with a trait such as defensive toxins that the host cannot make on its own (Jaenike *et al.* 2010; Pannicione *et al.* this volume) may then evolve varied levels of virulence and differing levels of mutualism towards the host depending on the strength and direction of interaction effects with other co-occurring symbionts within the host.

INTERACTIONS OF HOSTS AND SYMBIONTS – RED QUEENS OR RED HERRINGS?

Studies of pairwise symbiotic interactions have often sought evidence for antagonistic co-evolution as embodied in the Red Queen hypothesis (Van Valen 1973). But starting with an expectation of Red Queen dynamics may actually be a red herring, distracting us from the real issues of understanding evolutionary processes in the microbial communities within hosts. Returning to the two broad problems outlined in the introduction, we find the greatest need for theoretical work that explicitly incorporates ecological context-dependence (Thrall *et al.* 2007) and allows extensions to the diverse community of symbionts (Liow, Van Valen & Stenseth 2011) such as endophytes. Largely untouched is the possibility that the evolution of some species interactions may be more strongly affected by neutral ecological (Hubbell 2005) rather than selective processes because population sizes are small or the interactions occur too infrequently to generate the strong directional selection implied by arms race models. The importance of neutral forces does not necessarily run counter to the importance of selection in microbe–microbe, however, systems in which diversity is maintained by a constant flux of species should select for generalists defences, perhaps describing endophytes best. A clear incorporation of both ecological and evolutionary parameters will profitably focus on understanding the extent to which neutral ecological processes might drive the structure and function of endophyte communities.

One of the greatest challenges to empirical research agendas is to measure variation in fitness, and then genetic variation for fitness, difficult tasks in both simplified experimental systems (Gower & Webster 2005; Lee 2010; Lopez-Villavicencio *et al.* 2011) and in complex communities (Wade 2003, 2007). A reductionist approach will generate tractable genetic systems where trait associations can be evaluated, especially the traits affecting facilitation or competition between co-occurring microbes, and the traits affecting virulence towards the host. Outcomes that counter expectations from two-party systems are especially valuable. For example, Gower & Webster (2005) and Hughes & Boomsma (2004) demonstrate that a symbiont that is not aggressive towards the host can be an efficient competitor against a parasite. A community approach is required to determine whether community interactions result in qualitatively different fitness outcomes than predicted from simpler systems of two or three interacting organisms. We have but few examples (Rigaud, Perrot-Minnot & Brown 2010) and suggest that a productive avenue of research will be to first determine whether nonlinear fitness outcomes are obtained (Lello *et al.* 2004; de Roode *et al.*2011*)* and then to understand genetic variation in traits such as host resistance or microbial toxin production explaining variation in fitness outcomes. For example, results of Bell *et al.* (2006) show that initial infection by one parasite limits the host immune response to a second parasite. Further experiments might determine whether that outcome is achieved with differing numbers of species present. Rudgers *et al.* (2012) find a positive impact of a vertically transmitted endophyte on host population growth even though this defensive symbiont has negative effects on host survival. Further experiments might determine whether horizontally transmitted endophytes in this system evolve greater virulence towards the host, as predicted by the models of Lively *et al.* (2005). Lastly, aphids may change toxin composition of a plant host shared with butterflies sufficiently to drive greater virulence of a butterfly parasite (de Roode *et al.* 2011). As these three species examples differ significantly from pairwise expectations, further complexity will undoubtedly be uncovered by taking a community approach to studying symbiotic interactions.

In summary, we suggest that for endophytes, much of what we consider the 'defence' of defensive mutualisms may have evolved as a result of interactions with other microbes within the plant host. In this view, the protection of host resources, and possibly the evolution of greater virulence towards the host by a symbiont, may be an accidental bi-product of these often-cryptic ecological interactions. The expression of defence traits, while initially conditional, could become fixed in environments that provide strong selection for the maintenance of the trait (Fellous & Salvaudon 2009; Jaenike *et al.* 2010). In such cases, the host and defensive mutualist might co-evolve by any number of mechanisms: horizontal gene transfer to the host, compensation for the cost of supporting the mutualist, host tolerance to negative effects of the mutualist, or even a parasite that provides a necessary host function while also providing protection against other parasites. If our thesis is correct, endophytes used as biological control agents and parasites may adapt to each other, decrease the control effectiveness and possibly drive increased virulence by one partner towards the host (Duffy, Schouten & Raaijmakers 2003). Breaking through methodological and theoretical limitations to understand the evolution of symbioses in a community ecological context has important implications for effective biological control of agricultural plant enemies, as well as our understanding of the evolution of symbioses.

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