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The role of defensive symbionts in host–parasite coevolution

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ABSTRACT

Understanding the coevolution of hosts and parasites is a long-standing goal of evolutionary biology. There is a well-developed theoretical framework to describe the evolution of host–parasite interactions under the assumption of direct, two-species interactions, which can result in arms race dynamics or sustained genotype fluctuations driven by negative frequency dependence (Red Queen dynamics). However, many hosts rely on symbionts for defence against parasites. Whilst the ubiquity of defensive symbionts and their potential importance for disease control are increasingly recognized, there is still a gap in our understanding of how symbionts mediate or possibly take part in host–parasite coevolution. Herein we address this question by synthesizing information already available from theoretical and empirical studies. First, we briefly introduce current hypotheses on how defensive mutualisms evolved from more parasitic relationships and highlight exciting new experimental evidence showing that this can occur very rapidly. We go on to show that defensive symbionts influence virtually all important determinants of coevolutionary dynamics, namely the variation in host resistance available to selection by parasites, the specificity of host resistance, and the trade-off structure between host resistance and other components of fitness. In light of these findings, we turn to the limited theory and experiments available for such three-species interactions to assess the role of defensive symbionts in host–parasite coevolution. Specifically, we discuss under which conditions the defensive symbiont may take over from the host the reciprocal adaptation with parasites and undergo its own selection dynamics, thereby altering or relaxing selection on the hosts' own immune defences. Finally, we address potential effects of defensive symbionts on the evolution of parasite virulence. This is an important problem for which there is no single, clear-cut prediction. The selection on parasite virulence resulting from the presence of defensive symbionts in their hosts will depend on the underlying mechanism of defence. We identify the evolutionary predictions for different functional categories of symbiont-conferred resistance and we evaluate the empirical literature for supporting evidence. We end this review with outstanding questions and promising avenues for future research to improve our understanding of symbiont-mediated coevolution between hosts and parasites.

Key words: coevolution, defensive symbiosis, parasitism, microbial symbionts, mutualism, resistance, transmission modes.

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I. INTRODUCTION

All organisms are under constant threat of attack by pathogens and parasites, and as a result have evolved diverse strategies to avoid infection, including complex behaviours and sophisticated immune systems. In parallel, infectious agents are under strong selection to subvert host defences, and many of the best examples of ongoing coevolutionary interactions involve hosts and their parasites (e.g. Dybdahl & Lively, 1998; Decaestecker *et al.*, 2007; Gomez & Buckling, 2011).

Recently, there has been growing appreciation of another important arm in the arsenal of host defences – that of beneficial microbes. It is now clear that many organisms harbour microbial symbionts that protect them against natural enemies – this is currently one of the most active and exciting areas of symbiosis research (White & Torres, 2009; Clay, 2014; Ford & King, 2016). There is also much interest in using defensive symbionts as new control strategies to target parasites and pathogens of medical, economic and conservation importance. For example, *Aedes aegypti* mosquitoes transfected with a *Wolbachia* bacterial symbiont that is native to *Drosophila* fruit flies and that suppresses RNA viruses have recently been released in the wild, with the hope of combating Dengue (Schmidt *et al.*, 2017).

With the realization that defensive symbionts are common, it is important to understand how this third party affects the evolution of hosts and of the natural enemies they protect against. There is a large and sophisticated body of theory on host–parasite coevolution (reviewed in Woolhouse *et al.*, 2002; Salathé, Kouyos & Bonhoeffer, 2008), but most models of host–parasite interactions assume a direct interaction between host and parasite, without any interference from host-associated symbionts. Fortunately, this is slowly starting to change. Drawing on recent models of such tripartite interactions and exciting new empirical research, we try to consolidate how defensive symbionts mediate host–parasite coevolution. Specifically, we address the following questions: (*i*) do defensive symbionts coevolve with parasites; (*ii*) do defensive symbionts affect the evolution of the hosts' immune system; and (*iii*) do defensive symbionts influence the evolution of parasite virulence? As our main concern is on the consequences of defensive symbioses for host–parasite coevolution, we limit our discussion to symbiont-mediated protection against infectious organisms (i.e. parasites and pathogens). Thus, we do not specifically discuss symbionts that provide protection against predators, herbivores, and other grazers.

II. HOW DO DEFENSIVE SYMBIOSES EVOLVE?

Before addressing their consequences for host–parasite coevolution, it is useful to discuss how defensive symbioses arise. It is generally agreed that the evolution of mutualistic traits such as host protection is promoted by vertical transmission of symbionts (Ewald, 1987; Herre *et al.*, 1999; Sachs, Skophammer & Regus, 2011). However, vertical transmission is not a necessary precondition for the evolution of host defence. Theoretically, host defence or any other benefit to the host can also evolve under horizontal transmission if spatial structure generates covariance between host and symbiont genotypes, or if selection can act on more than additive benefits resulting from particular combinations of host and symbiont genotypes (interspecific epistasis) (Fitzpatrick, 2014). The same is true for any mechanism that entails a positive covariance between horizontal transmission and symbiont-provided benefits (Shapiro & Turner, 2014). This is the case, for example, if the host exerts adaptive partner choice for symbionts possessing particular traits, provides favourable conditions for symbiont growth, and then releases symbionts again, as seen in the symbiosis between the bobtail squid (*Euprymna scolopes*) and *Vibrio fischeri* (Nyholm & McFall-Ngai, 2004). Recent models by Ashby & King (2017) explore in more detail the conditions under which host protection can evolve in the case of multiple infections by parasites with horizontal transmission.

There is no question, though, that vertical transmission facilitates the evolution of host protection, because it makes the symbiont reliant on the successful reproduction of its host and thus aligns their evolutionary interests. It is therefore reasonable to assume that in many heritable

defensive symbionts we observe today, maternal transmission has evolved prior to host protection. Lively *et al.* (2005), drawing on earlier work by Lipsitch, Siller & Nowak (1996), used a simulation model to demonstrate that a vertically transmitted parasite can persist and spread in a host population if it provides protection against a more virulent horizontally transmitted parasite. Jones, White & Boots (2011) then showed that competition between a horizontally and a vertically transmitted parasite results in selection for host protection in the vertically transmitted parasite. Experimental corroboration of this prediction comes from an exciting new study employing experimental evolution in the laboratory to observe the *de novo* evolution of a defensive symbiont. King *et al.* (2016) allowed the mildly pathogenic bacterium *Enterococcus faecalis* in the gut of nematodes (*Caenorhabditis elegans*) to evolve either in the presence or absence of a competing, more virulent bacterium, *Staphylococcus aureus*. Worms were re-infected with *E. faecalis* from the previous worm generation, whereas *S. aureus* was re-supplied every generation anew from a frozen culture stock. Within only five host generations, *E. faecalis* evolved the ability to protect its host against *S. aureus* and strongly reduce *S. aureus*-induced host mortality. Mechanistically, host protection involved *E. faecalis* ramping up its production of antimicrobial superoxide (King *et al.*, 2016). The results from this nematode–bacteria system suggest that host protection is a trait that evolves relatively easily under the right ecological conditions, which is corroborated well by the rapidly increasing number of defensive symbioses described from natural systems (Florez *et al.*, 2015).

III. A CLASSIFICATION SYSTEM OF DEFENSIVE SYMBIONTS

Although there is enormous diversity of defensive symbioses, involving a wide range of host–parasite systems, there are a number of key features that are useful in classifying host–parasite–symbiont interactions, and in helping to make predictions about the long-term outcome of the association. Figure 1 illustrates how some of the best-known examples of protection can be visualized and classified.

(1) Transmission mode

As mentioned previously, how a symbiont is transmitted or acquired by its host plays an important role in the evolution of protection. Many of the best examples of defensive symbionts are transmitted either exclusively, or almost exclusively, from mothers to their offspring, over ecological timescales. For example, inherited bacterial symbionts have been shown to protect their insect hosts against parasitic wasps and nematodes, pathogenic fungi, and RNA viruses (e.g. Oliver *et al.*, 2003; Scarborough, Ferrari & Godfray, 2005; Teixeira *et al.*, 2008; Jaenike *et al.*, 2010). Vertical transmission promotes intimacy and stability of interaction between host and symbiont. The evolutionary interests of a host and its inherited symbiont are strongly aligned, and as the symbiont fitness is entirely dependent on its host's successful reproduction, it is under strong selection to counteract infectious organisms that reduce host fitness.

On the other hand, not all defensive symbionts are vertically transmitted. Many symbionts provide protection by colonizing host surfaces, such as plant leaves (Arnold *et al.*, 2003), amphibian skin (Harris *et al.*, 2009), or human gut epithelial tissue (Wexler *et al.*, 2016), preventing the establishment of pathogens. Theory predicts that this form of protection benefits from factors that promote stable interactions, for example, by increasing the probability that offspring surfaces will be rapidly colonized by symbionts of their parents. This may be promoted by specific mechanisms such as the maternal deposition of symbiont inoculates on eggs that are consumed by the hatching offspring (e.g. Kaltenpoth, Winter & Kleinhammer, 2009; Kaiwa *et al.*, 2014), but also more generally *via* social transmission or brood care. However, as most of these types of symbioses are part of complex microbial communities, little is known about stability and fidelity of transmission.

(2) Are defensive symbionts obligate or facultative for their hosts?

Almost all known defensive symbionts are facultative, meaning that although they cannot live independently from their host, the converse is not true, and the host does not typically require them for survival and reproduction. As a result, defensive symbionts do not typically form ancient associations with their hosts, nor do they cospeciate with them (e.g. Sandström *et al.*, 2001; Russell *et al.*, 2003). We are aware of a few examples where the defensive symbiont is obligate. Beewolves, which are wasps that hunt bees and use them to feed their young, contain specialized *Streptomyces* bacteria inside their antennae, that protect developing offspring from pathogenic fungi (Kaltenpoth *et al.*, 2005). All beewolves in the subfamily Philanthinae harbour these symbionts, and they have been stably maintained for 70 million years, although they have not cospeciated with their hosts, but instead have colonized new host species over evolutionary timescales (Kaltenpoth *et al.*, 2014). The Asian citrus psyllid (*Diaphorina citri*) harbours two obligate endosymbionts, *Profftella armaturum* and *Carsonella ruddi* (Nakabachi *et al.*, 2013). *Profftella* complements *Carsonella* by synthesizing the essential vitamins riboflavin and biotin, for which its co-symbiont lacks the required genes, and is thus likely to benefit the host nutritionally. In addition, a large portion of *Profftella*'s genome is devoted to producing a defensive polyketide toxin, although what natural enemy this protects against is not yet known.

It is highly challenging to demonstrate conclusively whether a specific member of a host surface microbiome, such as on amphibian skin, is obligate, as it is difficult to manipulate individual members of such a complex microbial community experimentally.

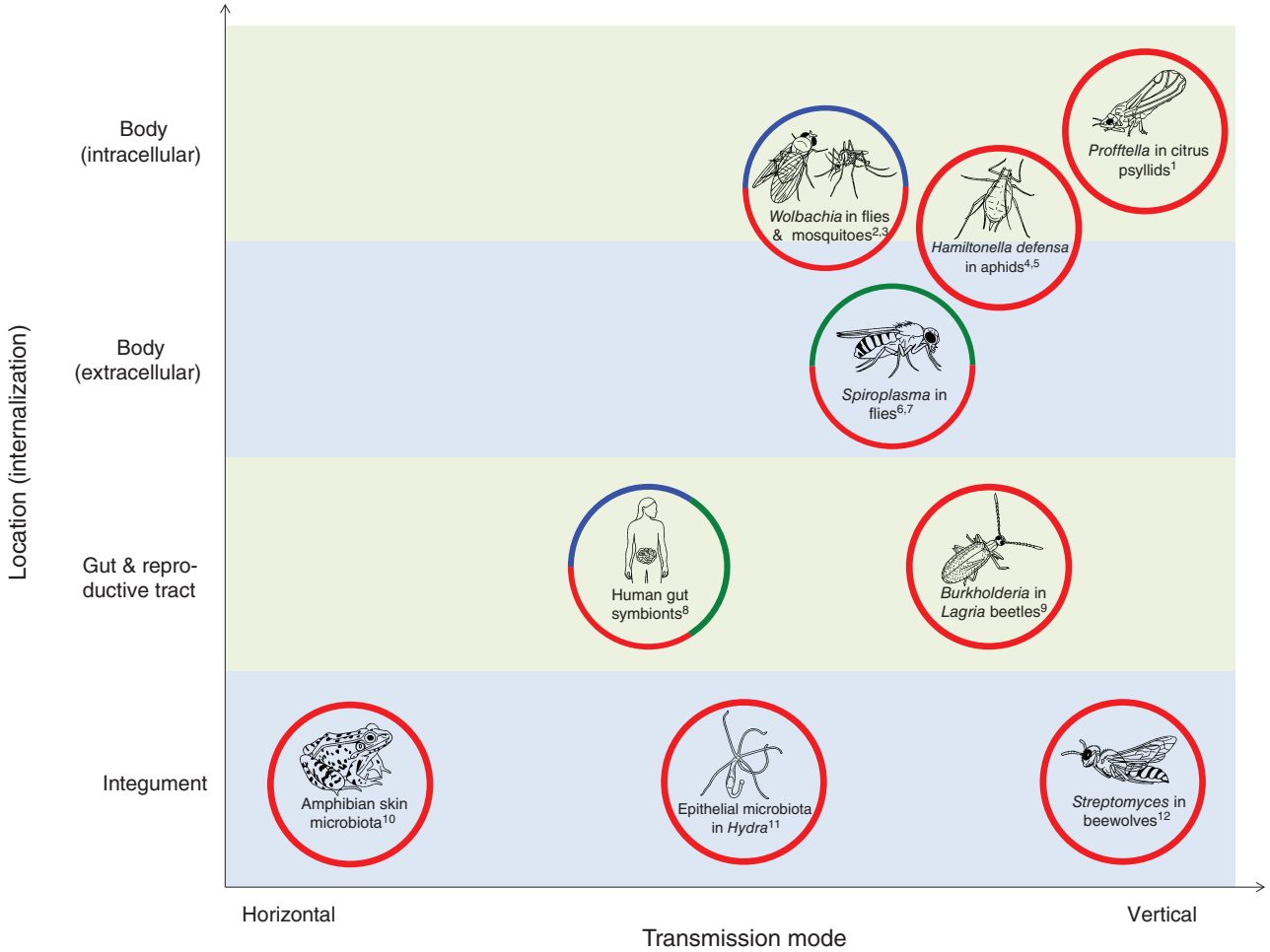

Fig. 1. A number of well-known defensive symbioses in animals, arranged along an axis from predominantly horizontal to predominantly vertical transmission of the symbionts, as well as an axis of increasing 'internalization', ranging from surface-colonizing to intracellular symbionts. The colours of the circles surrounding the organisms depict inferred mechanisms of symbiont-conferred protection: green – exploitative competition; red – interference competition (mostly *via* toxins); blue – apparent competition (*via* immune activation). References: [1]Nakabachi *et al.* (2013); [2]Teixeira, Ferreira & Ashburner (2008); [3]Moreira *et al.* (2009); [4]Oliver *et al.* (2003); [5]Schmid *et al.* (2012); [6]Jaenike *et al.* (2010); [7]Xie, Vilchez & Mateos (2010); [8]Honda & Littman (2012); [9]Flórez *et al.* (2017); [10]Harris *et al.* (2009); [11]Fraune *et al.* (2015); [12]Kaltenpoth *et al.* (2005).

(3) Location of the defensive symbiont in or on the body of its host

There is much variation in where protective symbionts occur in or on their hosts, and this is intimately tied with how the symbiont is transmitted, and what pathogens and other symbionts it has the potential to encounter and interact with. At one extreme lie intracellular endosymbionts of insects, with extracellular symbionts on host integumental surfaces at the other. While the transmission of intracellular symbionts is typically vertical, the transmission of symbionts colonizing host external and internal surfaces can range from predominantly horizontal to predominantly vertical (Fig. 1).

(4) Mechanism of protection

Finally, it is useful to consider how symbionts protect their hosts, although in general, this is still poorly understood. For this classification we follow earlier suggestions to distinguish three main, but not necessarily mutually exclusive, ways in which symbionts can provide protection. These all have parallels in the ecological literature on competition (Read & Taylor, 2001; Haine, 2008; Gerardo & Parker, 2014; Hamilton *et al.*, 2014). Species may compete *via* their joint demand for a limiting resource (exploitative competition), *via* direct interference with each other (interference competition), or indirectly *via* a shared natural enemy (apparent competition) (Wootton, 1994). The same mechanisms are applicable to the interaction between a defensive symbiont and a parasite sharing the same host (Fig. 2). A defensive symbiont may hinder the development of a competing parasite by depleting resources on which the parasite relies, by direct interference *via* the production of toxins, for example, or indirectly by activating the host's immune defenses, thus impeding further infections.

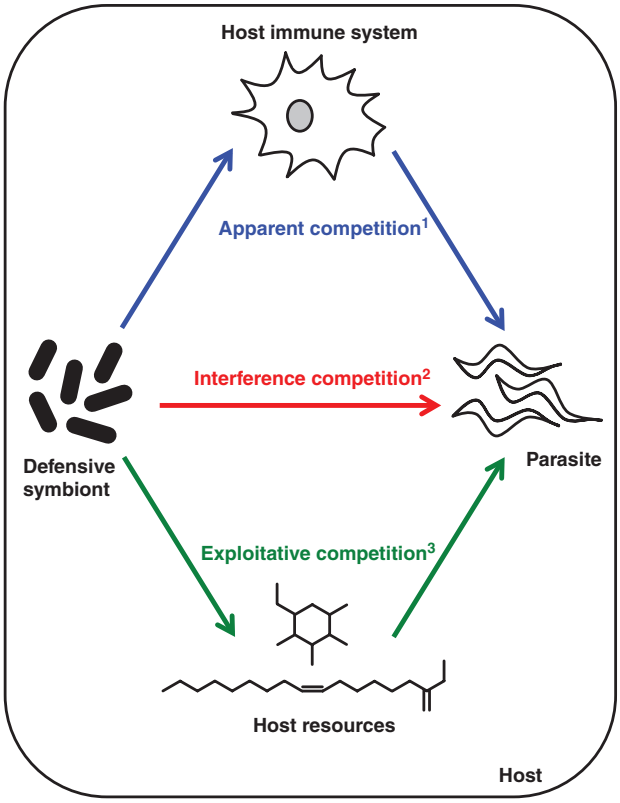

Fig. 2. Illustration of the three main mechanisms of symbiont-conferred protection against parasites. Examples: [1]in the mosquito *Aedes aegypti*, *Wolbachia* strain wAlbB (introduced from *Ae. albopictus*) induces increased levels of reactive oxygen species (ROS), thus activating the Toll pathway and increasing resistance to dengue virus (Pan *et al.*, 2012); [2]in *Drosophila neotestacea*, the endosymbiont *Spiroplasma* produces ribosome-inactivating proteins that play a major role in protection against parasitic nematodes and parasitoid wasps (Hamilton *et al.*, 2016; Ballinger & Perlman, 2017); [3]in *D. melanogaster*, competition for lipids is implicated in *Spiroplasma*-mediated protection against parasitoid wasps (Paredes *et al.*, 2016).

At first glance, exploitative competition between defensive symbionts and parasites seems almost unavoidable. Two organisms developing in the same host are likely to show at least some overlap in the resources they draw from the host. It is therefore surprising that well-documented examples of host protection *via* resource depletion are scarce. The most frequently cited example concerns the bacterial endosymbiont *Wolbachia*, which appears to block the growth of Drosophila C virus in *D. melanogaster via* competition for cholesterol (Caragata *et al.*, 2013). More recently, Paredes *et al.* (2016) reported that competition for lipids also plays a role in *Spiroplasma*-mediated protection against parasitoid wasps in *D. melanogaster*. The apparent scarcity of such examples might be related to the fact that defensive symbionts are often phylogenetically distant from the parasites they protect against (e.g. in cases of bacteria-mediated protection against eukaryotic macroparasites), whereas exploitative competition will certainly play a role in coinfections of closely related species or different strains of the same species. That said, we consider it likely that in many cases of protection ascribed to gut symbionts, sometimes referred to as priority effects (Wang & Rozen, 2017), competition for resources such as space and nutrients would certainly play a role.

Examples of host protection *via* interference are more commonly found in the literature. In several insects, the protection provided by defensive symbionts has been linked to the production of toxins targeting the parasite, e.g. in pea aphids (*Acyrthosiphon pisum*) protected against parasitoids by *H. defensa* (Oliver *et al.*, 2009), and in *D. neotestacea* protected by *Spiroplasma* against nematodes (Hamilton *et al.*, 2016) as well as parasitoids (Ballinger & Perlman, 2017). *Streptomyces* beewolf symbionts produce a complex cocktail of antibiotics to suppress pathogenic fungi (Kroiss *et al.*, 2010). Antibacterial toxins are also implicated in protection against pathogens by human *Bacteroides* gut symbionts (e.g. Wexler *et al.*, 2016). Toxins are thus a recurrent theme in defensive symbiosis. Considering that 'chemical warfare' is commonplace in competition among free-living microbes (Stubbendieck & Straight, 2016), it may not be so surprising that the same means are employed for competition within hosts.

Finally, symbionts can trigger a host immune response that suppresses parasites or pathogens – this is analogous to apparent competition. Immune priming has been reported in symbionts of bee guts (Kwong, Mancenido & Moran, 2017) and plant roots (Pieterse *et al.*, 2014), as well as strains of *Wolbachia* that have been transferred from flies into mosquitoes. The latter novel stable symbiosis results in upregulation of mosquito immunity genes that have been shown to suppress a wide range of infections, including Dengue and Chikungunya viruses, *Plasmodium* and filarial nematodes (Moreira *et al.*, 2009; Pan *et al.*, 2012; Rancès *et al.*, 2012). Interestingly, native fly and mosquito *Wolbachia* also suppress viruses, but without activating the host immune system (Wong *et al.*, 2011; Rancès *et al.*, 2012). Many bacterial endosymbionts of insects thus seem to 'stay under the radar' and remain unrecognized by the host immune system.

IV. THEORY OF HOST–PARASITE COEVOLUTION

To understand how defensive symbionts might influence the coevolution of hosts and parasites first requires a look at current theory of host–parasite coevolution, for which there is a well-developed mathematical framework (e.g. Anderson & May, 1982; Frank, 1994; Sasaki, 2000). It works under the well-supported assumption that host susceptibility to parasites is at least partially determined by host and parasite genotypes, which is implemented in the form of interaction loci. These are assumed genes in host and parasite that interact with each other to determine the outcome of a host–parasite encounter, originally inspired by the empirical observation of resistance–virulence

polymorphisms in crop plant–pathogen systems (Flor, 1971; Burdon, 1987). How these genes interact is determined by matrices that define the fitness of the host and parasite for each combination of host and parasite genotypes, according to a chosen interaction model (reviewed in Salathé *et al.*, 2008). Well-known canonical cases are the gene-for-gene (GFG) model, assuming that each resistance allele in the host needs to be countered by a 'virulence' allele in the parasite for a successful infection (Flor, 1956), or the matching alleles (MA) model (Frank, 1991), which requires an exact match of each host allele by a corresponding parasite allele for successful infection, in the style of the lock and key principle frequently encountered in immunology (Fig. 3). Note that in the plant pathology literature, where the GFG model was first applied, the term virulence was used to describe the ability to infect a host, i.e. parasite infectivity. We use the term 'infectivity' for this henceforth, to avoid confusion when we discuss the evolution of parasite virulence in the generally accepted sense as the parasite-induced fitness reduction of infected hosts.

The GFG and MA models were important for understanding the genetic dynamics driven by host–parasite coevolution, particularly in the context of the Red Queen hypothesis for the maintenance of sex and recombination (Jaenike, 1978; Hamilton, 1980; Peters & Lively, 1999). The high specificity of the interaction inherent in the MA model leads to rapid turnover of genotypes driven by negative frequency-dependent selection. A parasite genotype able to infect a common host genotype will be under positive selection and increase, eventually suppressing this host genotype such that another, previously rare host genotype can increase, only to meet the same fate when parasites able to infect it gain in numbers. This rapidly fluctuating selection has the potential to select for sex and recombination as a means to produce novel and rare genotypes (Peters & Lively, 1999). Under the GFG model, on the other hand, a universally infective parasite genotype is possible, such that a host–parasite system will see selective sweeps of host resistance and parasite infectivity alleles until eventual fixation at maximal infectivity in parasites and maximal resistance in hosts, barring further mutation (Parker, 1994). However, introducing the reasonable assumption that increased resistance and infectivity carry an intrinsic cost will also lead to negative frequency dependence and fluctuating selection dynamics in a GFG interaction (Frank, 1993; Parker, 1994; Sasaki, 2000) (Fig. 3).

The MA and GFG models are just two realizations of an effectively unlimited number of possible host–parasite interaction matrices. Agrawal & Lively (2002) argued that they can be seen as the endpoints of a continuum from extremely specialized parasites (MA – one parasite genotype fits one host genotype) to parasites with potentially broad host ranges (GFG – allowing for universally infective genotypes with infective alleles at all loci), and they went on to show that genotype fluctuations occur along most of this continuum. This was supported by formal analyses of generalized interaction models and by a comprehensive exploration of random interaction matrices (Engelstädter & Bonhoeffer, 2009; Engelstädter, 2015). A wide range of possible interaction models supports sustained cycles of genotype frequency fluctuations and thus maintains genetic variation in both antagonists, provided the interaction matrix exhibits some degree of genotype specificity, which can be quantified numerically (Kwiatkowski, Engelstädter & Vorburger, 2012).

At the risk of being overly simplistic, we summarize the theoretical work on host–parasite coevolution based on interaction loci as follows: Host–parasite coevolution may result in arms-race dynamics with reciprocal selective sweeps or in sustained genotype oscillations driven by negative frequency-dependent selection (Red Queen dynamics); the main determinants of the coevolutionary dynamics are (*i*) the genetic variation in host and parasite available to selection, (*ii*) the genetic specificity of the host–parasite interaction, and (*iii*) the costs or trade-offs associated with increased host resistance and/or parasite infectivity. We take this as a starting point to contemplate the potential effects of defensive symbionts on host–parasite coevolution.

V. DEFENSIVE SYMBIONTS AFFECT KEY DETERMINANTS OF HOST–PARASITE COEVOLUTION

Empirical research on heritable defensive symbionts, particularly those of insects, is yielding an increasing number of estimates of the strength and specificity of protection, but also of the costs to the host associated with harbouring these symbionts. Collectively, this work has shown that defensive symbionts have the potential to modify all of the main determinants of host–parasite coevolutionary dynamics.

(1) Heritable variation

To evolve in response to selection by parasites, host populations must possess heritable variation for susceptibility to these parasites. A straightforward way to estimate this variation is to compare infection success among multiple clones, full-sib families or half-sib families after a standardized exposure to parasites, using appropriate breeding designs to exclude confounding environmental effects. Numerous such experiments in various study systems have found significant differences among clones or families (e.g. Ebert, Zschokke-Rohringer & Carius, 1998; Ferrari *et al.*, 2001; Seppälä & Jokela, 2010; Lefevre, Williams & de Roode, 2011), providing evidence for heritable variation. Prior to the discovery of defensive symbionts, such estimates were necessarily naive to potential contributions of symbionts to the observed variation. This raises the question how much of the variation is encoded by the hosts' genomes, and how much is contributed by defensive symbionts. This cannot be determined in retrospect. Half-sib experiments in which the effect of dams is much higher than the sire effect might suggest an effect of maternally transmitted symbionts, but

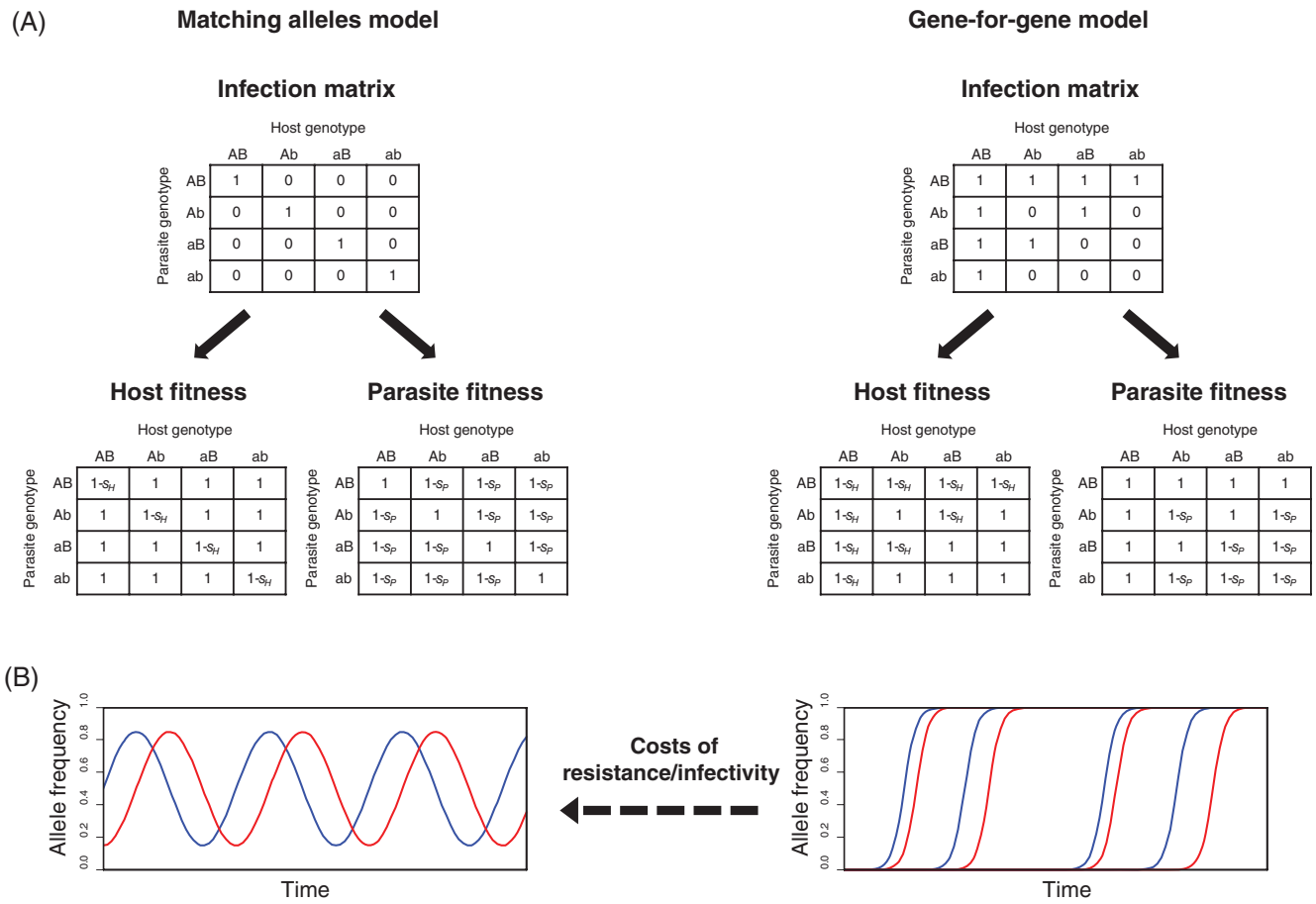

Fig. 3. Host–parasite interaction models and resulting genetic dynamics. (A) Illustration of two canonical cases of host–parasite interaction models, the matching alleles (MA) model and the gene-for-gene (GFG) model, assuming two biallelic interaction loci in a haploid system. The infection matrices describe which genotype combinations result in a successful infection. Under MA, an exact allelic match is required for infection to occur, while under GFG, each resistant allele in the host (lower-case letters) needs to be countered by an infective allele in the parasite (upper-case letters) for successful infection. These infection matrices result in the host and parasite fitness matrices below. The parameter s_H describes the cost of infection for the host, and s_p describes the cost of failing to infect for the parasite. (B) Illustration of predicted coevolutionary dynamics. Under a MA interaction, host (blue) and parasite (red) alleles are predicted to undergo time-lagged cyclical dynamics driven by negative frequency dependence, whereas a GFG interaction is predicted to result in successive selective sweeps of host and parasite alleles derived by mutation. The assumption of evolutionary costs to resistance and infectivity will also lead to negative frequency dependence and fluctuating selection dynamics under GFG (Sasaki, 2000; Agrawal & Lively, 2002).

maternal effects unrelated to symbionts may of course also affect resistance to parasites.

More recent experiments accounting for or manipulating the presence of defensive symbionts suggest that their contribution can be substantial. One good example is provided by the pea aphid. In an important early study on the evolutionary potential of host resistance to parasitoids, Henter & Via (1995) reported truly astounding variation among aphid clones, ranging from complete resistance to near-complete susceptibility. Only after Oliver *et al.* (2003) described aphid protection against parasitoid wasps by the abundant endosymbionts *Hamiltonella defensa* and *Serratia symbiotica* did it become clear that much of the resistance variation in pea aphids is due to variation in the possession of defensive endosymbionts rather than nuclear genetic variation among clones (Ferrari *et al.*, 2004; Oliver, Moran & Hunter, 2005; Bensadia *et al.*, 2006; Nyabuga *et al.*, 2010). The same can be said about pea aphid susceptibility to entomopathogenic fungi (Scarborough *et al.*, 2005; Łukasik *et al.*, 2013*a*,*b*). This does not mean, however, that there is no aphid-encoded resistance or that genetic variation is negligible in aphids. Considering resistance to parasitoid wasps, substantial and statistically significant clonal variation in the absence of defensive symbionts has been reported in several aphid species (von Burg *et al.*, 2008; Sandrock, Gouskov & Vorburger, 2010; A. J. Martinez *et al.*, 2014*b*), but defensive symbionts appear to augment the overall variation considerably. In the green peach aphid (*Myzus persicae*) or the black bean aphid (*Aphis fabae*), for example, clones harbouring defensive symbionts are much more resistant than the

most-resistant clones lacking these symbionts (von Burg *et al.*, 2008; Vorburger *et al.*, 2009). Laboratory experiments indeed confirmed that clones possessing heritable defensive symbionts are under strong positive selection in the presence of parasitoids (Herzog, Müller & Vorburger, 2007; Oliver *et al.*, 2008; Käch *et al.*, 2018).

Other insect systems provide a similar picture. The fungus-feeding fly *Drosophila neotestacea* gets parasitized by the sterilizing nematode *Howardula aoronymphium*, but infection with a heritable strain of *Spiroplasma* harms the nematodes and restores fly fertility (Jaenike *et al.*, 2010). This protection is stronger than any observed inherent variation in tolerance to the nematodes. It results in strong selection for *Spiroplasma*-infected flies in captive populations exposed to nematodes (Jaenike & Brekke, 2011), and it presumably explains the rapid spread of *Spiroplasma* in *D. neotestacea* across the North American continent (Jaenike *et al.*, 2010; Cockburn *et al.*, 2013). Other strains of *Spiroplasma* protect *D. hydei* and *D. melanogaster* against parasitoid wasps (Xie *et al.*, 2010; Xie *et al.*, 2014; Paredes *et al.*, 2016). Again, the resistance conferred by the defensive symbiont exceeds by far any variation observed among uninfected lines (Xie *et al.*, 2010), and the presence of parasitoids selects rapidly for flies protected by *Spiroplasma* (Xie *et al.*, 2015). Furthermore, flies of the genus *Drosophila* may benefit from protection against viral infection when they harbour *Wolbachia* (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). Also in this case, follow-up work showed that the effects of *Wolbachia* can be large relative to genetic variation within unprotected flies, and that among-strain variation in the strength of protection provided by *Wolbachia* may exceed even between-species variation of inherent resistance in flies (J. Martinez *et al.*, 2017*a*). Evolution in the presence of Drosophila C virus in *D. melanogaster* populations harbouring multiple strains of *Wolbachia* led to the rapid fixation of the most protective strain (Faria *et al.*, 2016).

Overall, the results from these different insect systems clearly suggest that polymorphism in infection with defensive symbionts augments heritable variation for resistance to parasites, and that the possession of defensive symbionts can be under strong positive selection in the presence of parasites. Unfortunately, there are virtually no data on genetic variation in defensive symbioses that are not vertically transmitted, such as gut and surface symbionts.

(2) Specificity

The genetic specificity of the interaction between hosts and parasites is of critical importance for the dynamics of their coevolution. Genotype-by-genotype interactions between hosts and parasites are conducive to negative frequency-dependent selection, resulting in rapid genotype fluctuations and the maintenance of genotypic variation in both antagonists (Woolhouse *et al.*, 2002; Schmid-Hempel & Ebert, 2003). A common approach to investigate specificity is infection matrix experiments. Multiple host lines are exposed to a number of different parasite strains in all possible combinations. In a specific interaction, the probability of infection will depend on the precise combination of host and parasite genotypes, which will be reflected in a significant statistical interaction. This approach has yielded clear evidence for genotype specificity from various study systems (e.g. Carius, Little, & Ebert, 2001; Schulenburg & Ewbank, 2004; Lambrechts *et al.*, 2005, 2009; Råberg *et al.*, 2014). In principle, this specificity can be mediated by traits encoded in the host and parasite genomes, by heritable endosymbionts, or both, but the role of symbionts has only been considered more recently (Vorburger *et al.*, 2009).

Meanwhile, a number of studies have shown that defensive symbionts can indeed contribute to or even be entirely responsible for the specificity of host–parasite interactions. A good example is provided by the black bean aphid and its parasitoid *Lysiphlebus fabarum*. This system allows for powerful tests of genotype specificity because asexual lines can be used for the host as well as the parasitoid (Sandrock & Vorburger, 2011). An infection-matrix experiment using multiple aphid clones that were free of defensive symbionts and multiple asexual lines of *L. fabarum* revealed significant among-line variation in aphid resistance and parasitoid infectivity, but no genotype-by-genotype interactions (Sandrock *et al.*, 2010). In a similar experiment using aphid clones possessing the defensive endosymbiont *Hamiltonella defensa*, on the other hand, genotype-by-genotype interactions were strong and highly significant (Rouchet & Vorburger, 2012). These interactions were recapitulated when genetically uniform aphids (a single clone) carrying infections with different strains of *H. defensa* were exposed to multiple asexual lines of the parasitoids (Schmid *et al.*, 2012; Cayetano & Vorburger, 2013, 2015), demonstrating that the specificity is due to a genetic interaction between the parasitoid and the bacteria protecting the host, rather than the host itself. Similarly, the protection against the entomopathogenic fungus *Pandora neoaphidis* conferred to pea aphids by the defensive endosymbiont *Regiella insecticola* is strongly dependent on genotype-by-genotype interactions between the symbiont and the pathogen (Parker *et al.*, 2017). Also in pea aphids, McLean & Godfray (2015) demonstrated specificity in the protection provided by *H. defensa* against different parasitoid species: strains protecting against one parasitoid species, *Aphidius ervi*, were ineffective against another species, *Aphelinus abdominalis*, and *vice versa*.

Gut symbionts can mediate the specificity of host–parasite interactions as well. The trypanosomatid *Crithidia bombi* is a virulent gut parasite of the bumblebee *Bombus terrestris* (Schmid-Hempel, 2001). Infection success depends on both the host colony (headed by a single queen) and the parasite strain (Mallon, Loosli & Schmid-Hempel, 2003; Sadd, 2011) – the hallmarks of genotype specificity. However, by transplanting gut microbiota among colonies, Koch & Schmid-Hempel (2012) showed that defensive gut symbionts are predominantly responsible for the specific resistance phenotypes.

Considering the limited number of pertinent studies, it is too early to decide whether this is a general phenomenon, but the above examples certainly indicate that defensive symbionts have the potential to increase the genetic specificity

of host–parasite interactions and thereby influence the reciprocal selection between the two antagonists.

The mechanism of symbiont-mediated protection is likely to be a major factor in determining the degree of specificity. For example, we might predict a high degree of specificity, as well as coevolutionary arms races, in symbioses involving polymorphic toxins. Although many defensive symbionts encode diverse toxins in their genomes, we are not aware of studies demonstrating that certain toxins are more or less effective against certain parasite genotypes, although circumstantial evidence exists (Dennis *et al.*, 2017). Specificity mediated by resource competition may be more difficult to achieve; we suggest that systems with rapidly evolving nutrient transporter genes, either in the symbiont, the host, or the parasite, may be useful places to look. Specificity in defensive symbioses involving the host immune system might be more likely in hosts with adaptive immunity, i.e. vertebrates, even though invertebrate immune systems can show specificity as well (e.g. Little *et al.*, 2003; Sadd & Schmid-Hempel, 2006). In another intriguing example of specificity mediated by a seemingly simple immune system, different species of *Hydra*, a lineage of cnidarians, encode species-specific antimicrobial peptides that shape the colonization of host-specific epithelial bacteria that provide protection against fungal pathogens (Franzenburg *et al.*, 2013; Fraune *et al.*, 2015).

(3) Costs and trade-offs

That defences against parasites come at a cost to the host is a central tenet of ecological immunology (Rolff & Siva-Jothy, 2003; Schmid-Hempel, 2003; Sadd & Schmid-Hempel, 2009). Costs of defence are important because they can maintain genetic variation for resistance under coevolution with parasites (Agrawal & Lively, 2002). It is reasonable to assume that resistance conferred by symbionts is also associated with costs, because the protection is provided by a population of symbionts that draws its resources from the host, and because some symbiont-provided defences (e.g. toxins) may not leave the host unaffected, leading to 'collateral damage'. This assumption is generally supported by empirical evidence from various study systems. For example, the defensive symbiont *H. defensa* reduces the lifespan and lifetime reproduction of its black bean aphid host in the absence of parasitoids (Vorburger & Gouskov, 2011). Indeed, infection with *H. defensa* seems to be counterselected in the absence of parasitoids, as shown by population cage experiments with pea aphids as well as cowpea aphids (*Aphis craccivora*) (Oliver *et al.*, 2008; Dykstra *et al.*, 2014). Protection against viral infection by *Wolbachia* in *D. melanogaster* also comes at the expense of a significantly reduced lifespan (Chrostek *et al.*, 2013; Martinez *et al.*, 2015). Costs of symbiont-conferred resistance need not be restricted to physiological costs that express themselves in negative effects on life-history traits. If defensive symbionts affect other traits, e.g. host behaviour, they can also entail ecological costs. For example, pea aphids protected against parasitoids by *H. defensa* show reduced defensive behaviour, resulting in a higher susceptibility to predators (Dion *et al.*, 2011*a*; Polin, Simon, & Outreman, 2014).

A relatively unexplored area is how defensive symbionts affect potentially costly host-encoded defences. Considering that they typically infect only part of the host population, defensive symbionts are unlikely to replace other defences. Also, there is clearly a need for the host to have some control over its microbiome, to 'keep it on a leash', as Foster *et al.* (2017) put it, and the immune system is a key instrument with which to exert this control, likely resulting in complex interdependencies. How defensive symbionts affect the evolution of the host immune system will further depend on the relative costs of these two lines of defence, and on whether their services are redundant or additive – something that is rarely addressed empirically (but see A. J. Martinez *et al.*, 2017*b*). An exciting new study by Martinez *et al.* (2016) indicates that defensive symbionts can relax selection on the host's own defences. They evolved replicate populations of *D. melanogaster* with and without a resistance-conferring strain of *Wolbachia* under selection by the Drosophila C virus for nine generations. Host resistance increased in both treatments, but the frequency of the resistant allele at the *pastrel* locus, a major effect gene for fly resistance to Drosophila C virus, rose to higher frequencies when flies were not protected by *Wolbachia*.

Costs may also explain why strong immune priming should be rare and/or short-lived in defensive symbiosis, because a permanently upregulated immune system would undoubtedly represent a significant cost to the host (Schmid-Hempel, 2003). Hence, it is to be expected that a long-term association between a host and a symbiont should favour the evolution of a state of low alert (Wong *et al.*, 2011). For example, defensive *Spiroplasma* do not trigger any detectable immune activation in *Drosophila* species, which may be due to the lack of a cell wall in *Spiroplasma* (Hurst *et al.*, 2003; Herren & Lemaitre, 2011). Likewise, native defensive *Wolbachia* do not change the expression of genes involved in innate immunity significantly, unlike novel strains transfected into mosquitoes (Rancès *et al.*, 2012, 2013).

In addition to their mere existence, the shape of trade-offs associated with protection has important implications for the coevolution of symbiont-possessing hosts and their parasites (Ashby & King, 2017). The evolutionarily simplest case would be that costs scale with protection, such that more strongly protective symbionts would also be more costly to the host in the absence of parasites. Such a relationship is to be expected if protection is a function of symbiont titre. *Wolbachia*-mediated protection against viruses in flies of the genus *Drosophila* appears to follow this pattern. The strength of protection provided by different strains of *Wolbachia* is directly related to their density in flies, but high *Wolbachia* titres also curtail fly lifespan (Chrostek *et al.*, 2013; J. Martinez *et al.*, 2014*a*, 2015). However, not all systems follow such a straightforward relationship. Different strains of *H. defensa* also vary in the strength of protection against parasitoids they provide to *A. fabae*, and all strains reduce lifespan and lifetime reproduction to some extent, but these costs are

inversely related to the strength of protection, such that the most protective strains are in fact least costly to the host (Cayetano *et al.*, 2015). It is important to note that this and similar inference is based on bivariate relationships under laboratory conditions (e.g. fecundity *versus* resistance), ignoring any other correlations or any ecological costs that could accrue in a more complex environment. The shape of the overall trade-off with protection against parasites might thus look different in wild populations, and this is potentially important. According to the model by Ashby & King (2017), the evolution of host protection is affected by whether the trade-off is accelerating or decelerating, which is something that could be difficult to estimate under realistic conditions, not to mention genotype-by-genotype interactions between host and symbiont, which may also affect the cost of protection (Vorburger & Gouskov, 2011). Nevertheless, the limited data available show clearly that defensive symbionts do have the potential to shape the relationship between resistance to parasites and other components of fitness, and thus to influence the dynamics of host–parasite coevolution.

VI. WHEN SYMBIONTS TAKE OVER AS DRIVERS OF COEVOLUTION

We have set forth above that defensive symbionts can underlie much of the variation in host resistance to parasites, that the interaction between defensive symbionts and the parasites they protect against can be genotype specific, and that defensive symbionts can mediate trade-offs between host resistance and other components of fitness. These findings imply a potential for dynamic coevolution between defensive symbionts and parasites, i.e. that defensive symbionts can be direct actors in tripartite (or multipartite) coevolution. That said, most of our theoretical understanding of host–parasite coevolution is based on models of pairwise interactions, since models of symbiont-mediated coevolution are still scarce, as are empirical data addressing the role of symbionts in host–parasite coevolution. Fortunately, the growing interest in defensive symbiosis has triggered some recent theoretical and empirical work that collectively supports an important role of defensive symbionts and helps understand their effect on coevolutionary dynamics.

In one of the first theoretical studies of tripartite coevolution in a host–symbiont–parasite interaction, Kwiatkowski *et al.* (2012) used a simple haploid genetic model with biallelic loci to investigate the resulting genetic dynamics, showing that when the symbiont–parasite interaction is more specific than the host–parasite interaction, the symbiont can engage in Red Queen dynamics with the parasite, essentially taking the coevolutionary interaction over from the host. A very recent theoretical study by King & Bonsall (2017) used a phenotypic approach to model a tripartite interaction, also allowing for epidemiological feedbacks on the densities of all three species. This model showed that defensive symbionts have a high potential to spread in an otherwise immune-defended host population, for which the negative effect of symbionts on parasite densities plays an important role, and it confirmed that defensive symbionts can be the central drivers of tripartite coevolution.

What about empirical support for coevolution between parasites and defensive symbionts? So far there is good evidence for counteradaptation of parasites to symbiont-conferred resistance in aphid–parasitoid interactions. Using experimental evolution, Dion *et al.* (2011*b*) demonstrated surprisingly rapid adaptation of the parasitoid wasp *Aphidius ervi* to the protection provided by *H. defensa* to its pea aphid host. Within only four generations of selection, parasitoid infectivity increased to the point that rates of parasitism were no longer different to those on unprotected hosts. Similarly rapid was the adaptation of the parasitoid *L. fabarum* to the presence of *H. defensa* in black bean aphids reported by Rouchet & Vorburger (2014), yet despite a significant improvement in their infectivity, parasitoids remained less successful than on unprotected hosts. This experiment employed three different strains belonging to two different haplotypes of *H. defensa*, and parasitoid adaptation turned out to be haplotype specific, such that adaptation to one haplotype did not improve parasitoid performance on the other, and *vice versa*. These are the prerequisites for negative frequency-dependent selection, which may result in fluctuating selection dynamics between parasites and defensive symbionts. Follow-up work has since shown that the different *H. defensa* haplotypes possess different toxin genes (Dennis *et al.*, 2017), which could feasibly represent different targets for parasitoid counteradaptation, although this functional link remains to be demonstrated. Interestingly, the specificity of parasitoid counteradaptation is also reflected in specific gene-expression changes of parasitoid wasps evolving with aphids possessing different strains of *H. defensa* (Dennis *et al.*, 2017).

An example of the opposite effect, symbiont adaptation to parasites, is provided by an experiment reporting the *de novo* evolution of a defensive symbiosis in *C. elegans*, where the gut bacterium *E. faecalis* was shown to adapt to the presence of pathogenic *S. aureus*, resulting in host protection (King *et al.*, 2016). Using an already established symbiosis, that between pea aphids and *H. defensa*, McLean & Godfray (2014) propagated individuals surviving a parasitoid attack for nine generations to test whether strong selection by parasitoids could lead to increased protection by the symbiont within a single aphid clone. However, no increase in protection was observed compared to control lines of the same clone that were not exposed to parasitoids. There are too many differences between these studies for a meaningful discussion of the unequal outcomes, but it is certainly to be expected that a long-established, natural symbiosis provides less variation for selection than a newly constructed association, and it is also possible that the bottleneck during vertical transmission in aphids further limits the opportunity for adaptation.

We are aware of only one experiment investigating the coevolution of defensive symbionts and parasites such that both antagonists were free to evolve. This experiment was

also carried out in *C. elegans*, by co-passaging the two gut bacteria *E. faecalis* (the newly evolved defensive symbiont) and *S. aureus* (the pathogen) for 10 worm generations (Ford *et al.*, 2017). Each microbe had the highest fitness when paired with antagonists from the recent past, providing evidence for reciprocal adaptation. Strong selection dynamics were further supported by frequency fluctuations of newly mutated alleles in both species.

The theoretical and empirical results summarized above are broadly consistent with reciprocal adaptation between defensive symbionts and parasites within the same hosts, and thus with a view of defensive symbionts as coevolutionary actors in their own right. However, mathematical models and laboratory experiments necessarily ignore some of the real-world complexities that may constrain coevolution of defensive symbionts and parasites. An important constraint is the high diversity of natural parasite communities. Most species are host to many different parasite species, some of which may be highly specialized, while others are generalists (Holmes & Price, 1986). *A priori*, symbionts are more likely to drive coevolution with host-specific parasites than with generalists that can move to other hosts, yet the need to defend against additional parasites may constrain this coevolution, especially if defences do not correlate across different enemies (e.g. Asplen *et al.*, 2014; Cayetano & Vorburger, 2015; McLean & Godfray, 2015).

A second complication is the environmental contingency that can be observed in symbiont-mediated protection. A good example is the thermal sensitivity of defensive symbionts (Corbin *et al.*, 2017). Extreme temperatures can affect their transmission as well as their function. For example, low temperatures have a negative effect on maternal transmission of *Spiroplasma* in *D. hydei* (Osaka *et al.*, 2008), whereas maternal transmission of *Wolbachia* in flies and booklice tends to fail at high temperatures (e.g. Hurst, Jiggins & Robinson, 2001; Jia *et al.*, 2009). In pea aphids, *H. defensa* is not eliminated by high temperatures, but protection against parasitoids begins to fail (Bensadia *et al.*, 2006), while the aphids' endogenous resistance remains unaffected by heat stress (Doremus *et al.*, in press), suggesting that the relative importance of symbiont and host contributions to defence is temperature-dependent. More generally, temperature will affect coevolution if it has unequal effects on different genotypes of interacting species (Thomas & Blanford, 2003), resulting in genotype-by-genotype-by-environment (G × G × E) interactions. This would entail that different genotypes are favoured under different thermal conditions. An experimental test in black bean aphids employing three different temperatures provided no evidence for G × G × E interactions between the defensive symbiont *H. defensa* and the parasitoid *L. fabarum* (Cayetano & Vorburger, 2013), but this may not be a general outcome.

The influences of the abiotic (e.g. temperature) and biotic environment (e.g. parasite community composition) on symbiont-conferred resistance are interesting research topics in their own right that deserve further investigation, but they also represent a challenge to detect signatures of symbiont-mediated coevolution in real-world situations.

VII. HOW DO DEFENSIVE SYMBIONTS AFFECT PARASITE VIRULENCE?

One of the most important questions related to defensive symbiosis is undoubtedly how defences conferred by symbionts affect the evolution of parasite virulence. Here we argue that a single clear-cut prediction is not possible because defensive symbionts may protect their hosts *via* different mechanisms, as discussed above. Alternative mechanisms can have different consequences for parasite virulence evolution. It is therefore useful to discuss each of the main mechanisms separately. Our discussion of their consequences for virulence evolution draws heavily on the theory of multiple infections (reviewed in Alizon, de Roode & Michalakis, 2013).

(1) The evolution of parasite virulence under resource/exploitative competition

If defensive symbionts and parasites interact *via* exploitative competition for resources, the expected consequences for parasite virulence evolution are probably least contentious. Several classical models predict an escalation of parasite virulence under within-host competition for resources (Bremermann & Pickering, 1983; Nowak & May, 1994; van Baalen & Sabelis, 1995; Frank, 1996), and this is supported by empirical evidence (de Roode *et al.*, 2005; Bell *et al.*, 2006; Ben-Ami, Mouton & Ebert, 2008). In the case of a vertically transmitted symbiont and a horizontally transmitted parasite, however, the two antagonists do not have equal stakes in competition for the host's resources. While the horizontally transmitted parasite is likely to gain fitness from increased virulence, the vertically transmitted one is more likely to lose fitness, because it relies on the host's survival and reproduction for its own transmission. The tripartite model of Jones *et al.* (2011) on the evolution of host protection by vertically transmitted parasites does indeed predict an increase in virulence only for the horizontally transmitted parasite. Given this imbalance in opportunities, it is worth considering that host protection predominantly *via* exploitative competition may be a transient stage in the evolution of defensive symbioses, because there should be strong selection on symbionts to evolve other means of harming the competing parasite. An interesting experiment in this context was done by Bashey, Hawlena & Lively (2013) on insect-parasitic nematodes of the genus *Steinernema*. These nematodes are associated with symbiotic bacteria of the genus *Xenorhabdus* that are required for successful infection and reproduction. Within the same host, different *Steinernema* species compete for resources but they may also compete by interference *via* bacteriocidal toxins (bacteriocins) produced by their *Xenorhabdus* symbionts. Mixed infections of different combinations in waxmoth (*Galleria mellonella*) caterpillars revealed that when both competitors possess

symbionts that do not produce bacteriocins, competition favoured the fastest killing (i.e. most virulent) nematodes, but in other combinations, less virulent nematodes were favoured when their symbionts produced bacteriocins able to inhibit the competitor's symbionts (Bashey *et al.*, 2013). Nevertheless, it remains a valid prediction that a heritable defensive symbiont can select for more virulent parasites if the two antagonists compete for host resources. This is an important concern when it comes to employing symbionts as a means to fight disease. There is increasing interest in introducing new defensive symbionts into natural populations of insect vectors to reduce their capacity to transmit harmful diseases such as dengue fever or malaria (Burt, 2014). Initial experiments were encouraging and field releases are underway (Hoffmann *et al.*, 2011; Iturbe-Ormaetxe, Walker & O'Neill, 2011). This approach holds great promise, but it will be important to understand the symbionts' mode of action in any such system so as not to select unwittingly for more virulent parasites (Jones *et al.*, 2011), which would clearly be undesirable.

A special and very different case of resource competition between parasites is the competition for 'public goods' produced by the parasites themselves. A good example is siderophores, compounds secreted by bacteria for the binding and uptake of iron (Wandersman & Delepelaire, 2004), which can also be exploited by cells other than the producers. For this reason, coinfections of unrelated bacteria will favour reduced siderophore production (Buckling *et al.*, 2007), and since siderophores are often important virulence factors of parasitic bacteria (Dale *et al.*, 2004), this special form of resource competition between parasites will lead to the evolution of reduced rather than increased virulence (West & Buckling, 2003; Harrison *et al.*, 2006).

(2) The evolution of parasite virulence under interference competition

Host protection by interference appears to be the most commonly documented mode of action of defensive symbionts (Ford & King, 2016). If we turn again to the theory of multiple infections as a first point of orientation (Alizon *et al.*, 2013), there is an obvious link to spite, i.e. actively decreasing the fitness of the competing parasite (Gardner, West & Buckling, 2004). Spiteful interactions are costly. In bacteria, the production of bacteriocins against competing bacteria is associated with a slower growth rate (reviewed in Riley & Wertz, 2002), and so is resistance to bacteriocins produced by competitors (Dykes & Hastings, 1998; Feldgarden & Riley, 1999; Gravesen *et al.*, 2002). This leads to the prediction that parasite competition *via* interference should lead to the evolution of reduced virulence, which is indeed supported by a number of studies (e.g. Massey, Buckling & ffrench-Constant, 2004; Inglis *et al.*, 2009; Garbutt *et al.*, 2011). It appears that this prediction can be extended to the case of defensive symbiosis. If resident defensive symbionts protect their host against parasites by interference, e.g. *via* the production of toxins, counteradaptation by parasites would either require the evolution of some form of resistance to these toxins, or their own production of anti-symbiont compounds to suppress the defender. Either will be costly for the parasites and thus likely reduce their virulence.

This prediction is testable by experimental evolution, and a recent study by Ford *et al.* (2016) does indeed support this prediction, albeit with a twist. It employed the *de novo* defensive symbiosis between *C. elegans* and the gut bacterium *E. faecalis*, which evolves to protect the worms against the more virulent bacterium *S. aureus* through increased production of antimicrobial superoxide (King *et al.*, 2016), as described in more detail in Section II. When *S. aureus* was cycled through *C. elegans* for 10 worm generations together with *E. faecalis*, it evolved a lower virulence compared to when it was cycled through worms alone. However, the inferred reason for the reduced virulence was not an increased tolerance to superoxides, instead it was related to a lower production of siderophores (see Section VII.1). The defensive symbiont *E. faecalis* exploited and benefitted from the *S. aureus*-produced siderophores (a 'public good'). This selected for lower siderophore production by *S. aureus*, which was in turn related to a lower virulence of *S. aureus* in worms (Ford *et al.*, 2016). This confirms that public good competition can have very different consequences for parasite virulence than competition for resources provided by the host. We are not aware of any other experimental evolution studies investigating the effects of defensive symbionts on parasite virulence under interference competition, but such studies are certainly feasible in other systems as well.

(3) The evolution of parasite virulence under immune priming

Predictions for the evolution of parasite virulence are least straightforward when host protection by symbionts involves apparent competition *via* the host's immune defences. An important aspect to consider is the specificity of the host immune response. It has been argued for mixed-strain infections of parasites that relatively generic responses should select for increased virulence (Råberg *et al.*, 2006). This prediction is based on the assumption that the strength of the immune response scales with parasite density. A more virulent strain would thus induce a stronger immune response than a less virulent strain, such that the less virulent strain would suffer more from competition with the more virulent strain than *vice versa*. The prediction changes if the host immune response is highly specific and the immune system directs its response mainly at the most abundant strain. Under these conditions, a strain with a low exploitation and growth rate might partly escape the notice of the immune system and do better in a mixed infection than on its own, which should select for reduced virulence. In an experimental test competing two strains of rodent malaria in immunocompetent and immunodeficient mice, the competitive suppression of the less virulent strain was slightly stronger in immunocompetent mice, suggesting weak selection for higher virulence under immune-mediated competition (Råberg *et al.*, 2006).

It is difficult to extend these results to defensive symbioses. Most of the examples we discuss above concern invertebrates with immune responses that are considered to be less specific than those of vertebrates (Pancer & Cooper, 2006), and they suggest that defensive symbionts select for more virulent parasites. In this context it is tempting to draw a parallel between defensive symbionts and 'imperfect vaccines', which also select for increased virulence (Gandon *et al.*, 2001; Mackinnon, Gandon & Read, 2008).

On the other hand, invertebrate immune defences can show a significant degree of specificity (e.g. Little *et al.*, 2003; Sadd & Schmid-Hempel, 2006; Pham *et al.*, 2007; Roth *et al.*, 2009), and defensive symbionts are often very different from the parasites they protect against (e.g. endosymbiotic bacteria protecting against fungi and parasitoid wasps). Hence they may be targeted by different weapons of the host immune system, which should not select for increased virulence. Furthermore, if parasite counteradaptation to the presence of defensive symbionts requires the evolution of an increased tolerance to the host immune response triggered by the symbiont, this might well be costly to the parasite and possibly result in reduced virulence.

The above arguments suggest that the evolutionary consequences of immune-mediated protection for parasite virulence might depend on the particular organisms involved. We are optimistic that there will soon be an empirical answer at least for some cases. Host protection *via* immune activation has been found in study systems that are experimentally very tractable, such as mosquitoes (e.g. Pan *et al.*, 2012). They provide an opportunity to employ experimental evolution of parasites in the presence and absence of defensive symbionts to examine the effects on parasite virulence.

(4) Symbionts and tolerance

In addition to resisting infection, another important way that hosts can defend against the negative fitness consequences of parasites is by tolerating them (Medzhitov, Schneider & Soares, 2012). Although tolerance has received much less attention than resistance, it is a common and important host strategy (Kover & Schaal, 2002; Råberg, Sim & Read, 2007). Tolerance has very different evolutionary outcomes than resistance, because it does not result in lower parasite fitness (Roy & Kirchner, 2000; Svensson & Råberg, 2010). As a result, tolerance does not impose selection on parasites to counter-resist hosts, or to increase virulence. Furthermore, a number of studies have found negative correlations (or trade-offs) between host tolerance and host resistance (Koskela *et al.*, 2002; Råberg *et al.*, 2007).

As far as we are aware, there are very few examples of symbionts that protect their hosts by increasing tolerance to infections. Here, symbiont removal would result in reduced host fitness, as well as negative (or no) fitness effects on parasites. Symbiont-mediated tolerance could occur in a number of ways (Ayres, 2016). For example, a symbiont could detoxify or consume a metabolite that is produced as a by-product of parasite growth and that is harmful to the host. Another example might result from an interaction between host immunity and resource competition between symbiont and parasite. Symbionts might preferentially colonize a sensitive host tissue, restricting parasite growth to other parts of the body. Removal of the symbiont would allow parasite colonization that might trigger a costly inflammatory immune response that is harmful to both host and parasite. The human gut microbe *Bacteroides fragilis* produces a factor, polysaccharide A (PSA), that protects against the harmful effects of *Helicobacter hepaticus*, although it does not reduce titres of *H. hepaticus* (Mazmanian, Round & Kasper, 2008). Instead, removal of *B. fragilis*, or of PSA *via* targeted gene knockout, results in an inflammatory response and colitis, triggered by host cytokine production in response to colonization of *H. hepaticus*. An important future direction will be to uncover more cases of symbiont-mediated tolerance; these are certain to play important roles in shaping the trajectory of coevolution and virulence.

The concept of tolerance is more difficult to apply to parasites that need to kill their hosts to complete development, like parasitoid wasps or some pathogenic fungi. Such parasites need to be resisted to rescue host fitness fully, although strategies to maintain reproduction of infected individuals prior to parasite-induced death, e.g. avoiding castration by the developing parasite, could be interpreted as a form of tolerance to lethal parasites. There is even evidence for increased or hastened host reproduction in response to infection by parasites (e.g. Chadwick & Little, 2005; Vale & Little, 2012; Leventhal, Dünner & Barribeau, 2014), referred to as fecundity compensation, but there are currently no data suggesting that defensive symbionts are involved in any of these effects.

VIII. OUTSTANDING QUESTIONS

The theoretical and empirical work on defensive symbioses reviewed above supports an important role of symbionts in mediating host–parasite coevolution. However, the empirical support comes predominantly from laboratory experiments that minimize environmental complexity (see Section VI). Demonstrating the importance of symbiont-mediated coevolution in natural populations is a challenge that remains to be addressed. What kind of data or experiments would be required for a convincing demonstration?

Patterns of local adaptation could provide a first indication. For example, Hansen *et al.* (2007) reported that the frequencies of a suspected defensive symbiont in the red gum lerp psyllid (*Glycaspis brimblecombei*) were positively correlated with the risk of wasp parasitism. On the other hand, aphid parasitoids from sites with a high prevalence of *H. defensa* in their hosts were not significantly better at overcoming *H. defensa*-conferred resistance than parasitoids from sites with a lower prevalence (Vorburger & Rouchet, 2016). Pervasive local adaptation of one antagonist is not necessarily expected under time-lagged, fluctuating selection dynamics, because local populations may be in different phases of reciprocal adaptation, making this type

of comparative evidence inconclusive (Kawecki & Ebert, 2004). Therefore, time-shift experiments (Gaba & Ebert, 2009), carried out with field-collected strains of parasites and defensive symbionts, would be a more promising approach. For some host–parasite systems it should be possible to collect strains of parasites and defensive symbionts from a natural population over multiple time points and maintain them in the laboratory without selection. Mutual exposures of these strains across different time points could then be used to test for reciprocal adaptation. Patterns paralleling the outcome of laboratory coevolution studies, supporting time-lagged adaptation between antagonists (e.g. Brockhurst *et al.*, 2003; Ford *et al.*, 2017), would make a strong case for an active role of defensive symbionts in host–parasite coevolution.

Another important question to address in the future is the evolutionary potential of defensive symbionts relative to that of the parasites they protect against. This becomes particularly important when symbionts are employed to combat disease. At first glance, the introduction and release of a defensive symbiont in host or vector populations is a more sustainable strategy than the introduction of a new drug. A drug is a standing target for parasite counteradaptation, whereas defensive symbionts can coevolve with the parasites. But who has the upper hand in this coevolutionary arms race? If a Dengue virus-suppressing strain of *Wolbachia* can be introduced successfully in mosquito populations transmitting the disease, for how long will this strategy work?

The evolutionary potential of parasites and symbionts will be determined by their population sizes, generation times and mutation rates. In the case of microbial symbionts protecting against macroparasites such as parasitoid wasps, the advantage may well lie with the rapidly replicating symbionts, which occur in populations of many millions in every host (e.g. Schmid *et al.*, 2012; Herren *et al.*, 2014). The situation might be reversed, however, in the case of symbionts protecting against viral pathogens with enormous population sizes and high mutation rates (Holmes, 2009). Moreover, the effective population size of microbial symbionts with vertical transmission is likely to be reduced severely by transmission bottlenecks between mothers and offspring (O'Fallon, 2008), and the compartmentalization into host individuals effectively generates a metapopulation structure that may reduce the long-term effective population size further (Whitlock & Barton, 1997), although there are conditions under which genetic variation at the metapopulation level may remain high (Harrison & Hastings, 1996). It appears that the relative evolutionary potential of parasites and defensive symbionts is not a straightforward problem. It might be easier to address with mathematical models than empirically, but ultimately it will be of great practical importance when it comes to using symbionts against (coevolving) parasites.

IX. CONCLUSIONS

(1) Microbial symbionts are ubiquitous and protection against parasites is a repeatable evolutionary outcome of a microbe's stable association with a eukaryotic host.

(2) The presence of defensive symbionts can alter the reciprocal selection between hosts and parasites by modifying key determinants of coevolutionary dynamics, such as the variation, the specificity and the cost of resistance.

(3) Defensive symbionts can be active, partially independent players in a tripartite interaction and sometimes take over from the host the coevolutionary arms race with parasites.

(4) The possession of defensive symbionts can relax selection on the host's own defences, and it can shape the evolution of parasites, particularly their virulence.

(5) It will be important to consider the evolutionary consequences of symbiont-conferred protection as research on defensive symbioses moves from the laboratory benches of curious scientists to field applications for disease control.

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