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Toxin-mediated protection against natural enemies by insect defensive symbionts

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Abstract

All organisms have evolved a wide range of strategies to protect against natural enemies, from complex avoidance behaviors to sophisticated immune systems, and insects are no exception. A more recently recognized, and likely widespread, mode of protection for insects is through the use of microbial symbionts. Many of the best studied examples of defensive symbiosis in insects involve infections with maternally inherited symbionts. While inherited symbionts can protect their insect hosts by priming innate immune systems, or competing with enemies for scarce resources, they have also been found to synthesize a wide diversity of toxins that effectively kill, weaken, or deter parasitic wasps, parasitic nematodes, pathogenic fungi, and predatory spiders. In this chapter, we provide an overview of defensive symbioses in insects, focusing in particular on toxin-based protection.

Keywords: insect, *Hamiltonella*, polyketide, symbiosis, toxin, *Spiroplasma*, *Wolbachia*

Introduction

Insects are key components of terrestrial ecosystems, often functioning as the dominant herbivores, decomposers and natural enemies in a given community. These diverse functional roles are often aided by the presence of resident microbes, which can bring novel capabilities that complement existing host function or provide novel functions to the host (Douglas 2015, Engel

and Moran 2013, Feldhaar 2011, Frago et al. 2012, Hansen and Moran 2014, Oliver and Russell 2016). The basis for the establishment, and often long-term persistence of insect-microbe associations is simply that the latter encode far greater biosynthetic potential as a group relative to the former (Moran 2007, Smith 1989). Hence while partnering with microbes may incur both short- and long-term costs, the acquisition of new or improved functions via symbiont infection may also bring net fitness benefits that stabilize specific insect-microbe associations. While widespread roles for symbionts in degrading plant polymers and provisioning limiting nutrients have long been recognized (Baumann 2005, Breznak and Brune 1994, Brune 2014, Buchner 1965), the past fifteen years have also seen the emergence of defensive symbiosis as a common phenomenon in insects (Ballinger and Perlman 2019, Brownlie and Johnson 2009, Florez et al. 2015, Van Arnam et al. 2018, Vorburger 2014). Perhaps this is not surprising given that microbes, especially bacteria, are well known for producing diverse, potent toxins that harm animal tissues or other microbes (Florez et al. 2015, Van Arnam et al. 2018), combined with the shared threat of enemy attack, regardless of feeding niche. Of course, insects have evolved a range of behaviors and endogenous physiological defenses, both cellular and humoral, to avoid or thwart attack from enemies (Gross 1993, Lemaitre and Hoffmann 2007, Strand 2008). However, many insects also leverage microbial infections to aid in their defenses, including the deployment of tactics that incorporate microbe-encoded toxins, which will be the focus of this review.

Insect-microbe Symbioses

Insect tissues exposed to the environment, including the cuticle and gut, are routinely colonized by microorganisms. Cuticular symbionts are often involved in pathogen defense, while gut symbionts may facilitate nutrient acquisition, degrade harmful compounds or protect against ingested pathogens (Douglas 2015, Engel and Moran 2013). Gut associated-microbes persist extracellularly and are typically environmentally transmitted (e.g. acquired in diet and excreted) with microbial diversity and specificity, as well as any host specialization (e.g. symbiont crypts in midgut), being highly variable across taxa and depending on the environment. Other microbes are largely or entirely restricted to the tissues or cells of their insect hosts. These ‘endosymbionts’ often, but not always, persist intracellularly and are transmitted vertically from

61 mothers to eggs or embryos (Moran et al. 2008). By definition, such heritable symbionts are
62 specialized to the symbiotic lifestyle, although to differing degrees. Some associations are
63 mutually obligate in which the symbiont(s) and host each require the other for survival and
64 reproduction. These tend to be ancient, highly-specialized associations where specific bacteria
65 are sequestered into host cells called bacteriocytes and involve sophisticated metabolic
66 coordination (where studied) between partners (Douglas 2014, Feng et al. 2019, Moran et al.
67 2008, Wilson and Duncan 2015). For example, all plant sap-restricted (i.e. xylem or phloem)
68 herbivores house bacteriocyte-associated symbionts that provision amino acids and co-factors
69 that occur in insufficient quantities in their diets (Baumann 2005, Buchner 1965). Past symbiont
70 infections enabled the use of plant-sap as a niche, which in turn facilitated diversification of
71 aphids, cicadas and other sternorrhynchan and auchenorrhynchan hemipterans. The genomes of
72 bacteriocyte-associated symbionts are highly reduced, often tiny, compared to free-living
73 bacteria but tellingly retain genes for the biosynthesis of key nutrients (Moran et al. 2008,
74 Shigenobu et al. 2000). This repeating pattern combined with the mere presence of these
75 symbionts in all individuals of all species feeding on nutrient-restricted substrates, provides
76 compelling correlational evidence of the generality of these nutritional mutualists, although few
77 experimental studies confirm these roles (Buchner 1965, Gunduz and Douglas 2009). One
78 nutritional symbiont, *Profftella armatura* (Betaproteobacteria), also encodes a polyketide toxin
79 called diaphorin that potentially functions in defense (Nakabachi et al. 2013) and is discussed in
80 more detail below.

81 A second class of heritable symbionts are facultative from the host perspective, meaning they are
82 not generally required for host survival, although insect survival may be contingent on their
83 presence under some conditions (Oliver et al. 2014). Heritable facultative symbionts are very
84 common, arguably infecting most insect species, although distributions both within and among
85 taxa are highly variable (Moran et al. 2008, Weinert et al. 2015). While most facultative
86 symbionts persist intracellularly, they can vary substantially in the tissues they inhabit, which are
87 determined by host and symbiont genotypes. The maternal inheritance patterns of facultative
88 symbionts provide two routes for symbiont spread and persistence within host populations: 1)
89 they can spread by providing net fitness benefits to hosts, 2) manipulate sexual reproduction in
90 ways that favor the spread of infected females (Werren and O'Neill 1997). In other words, if
91 heritable facultative symbionts are present at moderate infection frequencies, then they are likely

having important effects on host biology. Most of the early work on heritable facultative symbionts focused on reproductive manipulators as their symbiont-mediated phenotypes (e.g. biased sex ratios) were often conspicuous. Theoretical and experimental work identified four major types of reproductive manipulation: a) male-killing, where the sons of symbiont-infected females die early in development, b) feminization, where symbiont-infected genetic males are transformed into phenotypic females that can transmit symbionts, c) parthenogenesis-induction, where symbiont-infected females reproduce clonally, and d) cytoplasmic incompatibility, where matings between symbiont-infected males and uninfected females produce virtually no offspring, resulting in a relative benefit to symbiont-infected females (Werren and O'Neill 1997). The bacterial lineages associated with these strategies, including *Wolbachia*, *Cardinium*, *Spiroplasma*, *Rickettsia*, and *Arsenophonus*, infect a broad range of insect taxa (Duron et al. 2008). Many of these same species were subsequently found to perform beneficial roles, especially in host defense (e.g. (Hedges et al. 2008, Jaenike et al. 2010, Teixeira et al. 2008, Xie et al. 2014)). In addition, other facultative symbiont species not previously associated with reproductive manipulation were also found to confer benefits to hosts. These include defense against thermal stress, pathogens, parasites, parasitoids and predators, as well as expansion of dietary breadth and other interactions with food plants (Hansen and Moran 2014, Oliver and Moran 2009). Most, but not all, defensive symbionts characterized in insects are heritable, facultative symbionts, so they will be the focus of this review.

Defensive symbionts.

Insect are attacked by a range of microbial pathogens (viruses, bacteria, fungi and eukaryotic microbes), parasites (especially nematodes), parasitoids (especially hymenopteran wasps), and predators from diverse taxa. While variable across groups, common defenses include avoidance and escape behaviors, physical barriers, as well as cellular and humoral responses when barriers are breached (Lemaitre and Hoffmann 2007). Cellular responses include the phagocytosis of invading pathogens and the encapsulation and asphyxiation by hemocytes of larger invaders, including parasitoids (Strand 2008). Humoral defenses involve the production of inducible bioactive factors including antimicrobial peptides (AMPs) that may target specific groups of bacteria and fungi (Sheehan et al. 2018, Zasloff 2002). In some groups, more specialized and sophisticated endogenous responses have evolved, including the chemical explosions of bombardier beetles and the cantharidin toxins of blister beetles (Nelsen et al. 2014).

To bolster endogenous defenses, insects may benefit from symbiont infections in three, not mutually exclusive ways (Haine 2008). First, infection with heritable symbionts may prime the innate immune system such that responses are stronger upon subsequent enemy challenge. For example, some strains of *Wolbachia* that have been artificially transfected from *Drosophila* into mosquitoes upregulate host immunity pathways, effectively suppressing a range of vectored human pathogens, including Dengue, Zika, Chikungunya, West Nile virus and *Plasmodium* (Caragata et al. 2019). A second means by which symbiont infection can provide host protection is via direct competition with natural enemies for limiting resources. In *Drosophila melanogaster*, *Wolbachia* is reported to compete with *Drosophila C* virus for cholesterol (Caragata et al. 2013), while the symbiont *Spiroplasma* competes with internally-developing parasitoids for lipids (Paredes et al. 2016). While some level of resource competition between facultative symbionts and eukaryotic enemies is inevitable, the relatively low abundance of the symbionts, together with often varying tissue tropism and phylogenetic distance between them suggests this mode may not generally produce large effects. However, small effects may tip the precarious balance in the host-parasite tug-of-war, and may serve as an adjuvant for other means of symbiont-protection. For example, limiting resources, such as cholesterol or lipids (Caragata et al. 2013; Paredes et al. 2016) may serve to trigger the immune system, or may be involved in packaging or delivery of toxic symbiont-derived factors.

Finally, as a third strategy, hosts may employ the arsenal of bacterial encoded toxins to their own benefit. Bacteria synthesize an astonishing range of secreted factors that harm other entities. In free-living bacteria, toxins mediate competition with other microbes and protect against predators, while in pathogenic bacteria, toxins may harm host tissues, subvert host immune responses, or harm competing microbes. Most are proteins that are synthesized within the cell and exported to targets (exotoxins), but others, lipopolysaccharides (LPS), are membrane associated compounds released after cell disruption (endotoxins) (Kumar et al. 2019). Among the most potent compounds characterized, bacterial toxins produce virulence via a wide range of activities, including inhibition of DNA/protein synthesis, activation or inhibition of adenylate cyclase, pore formation or cell lysis, and modification of signaling pathways (Kumar et al. 2019, Schmitt et al. 1999). In general, toxin production presumably benefits the bacterial pathogen as enhanced virulence is expected to increase opportunities for replication and transmission. It is also useful to distinguish between toxin factors that are employed to enter and affect eukaryotic

cells, as opposed to prokaryotic ones.

Several symbionts from diverse insect taxa use microbial toxins to kill parasites, parasitoids and predators. Although there are still only a handful of documented cases, employment of toxic factors is probably the most common mechanism of symbiont-mediated protection. This may perhaps be because, similar to qualitative versus quantitative defenses, concepts that are especially well-developed in the literature on plant defenses against herbivores (Feeny 1975; Coley et al. 1985), toxins may be less expensive for the host than global stimulation of the immune system or depletion of limiting resources.

For the remainder of this chapter, we will discuss in detail the known examples of protection involving production of toxic factors by inherited symbionts. We have attempted to restrict our discussion to cases where there is an especially strong connection between the symbiont, defense, and a specific toxic factor (see also Table 1 for an overview of the specific examples we review). In some cases, however, it has not yet been demonstrated whether protection is occurring in nature, and against whom. This highlights the challenges in studying defensive symbiosis in nature (Oliver et al. 2014) as it requires a good understanding of both an insect's symbionts and ecologically relevant natural enemies. We have also restricted our discussion to toxins where there has been some functional work directly linking them to protection. With the revolution in genome sequencing, we are finding that many symbiont genomes, especially those of facultative symbionts, harbor a great diversity of genes with sequence similarity to toxins that have been characterized in other microbes, but we have no idea whether the putative symbiont factor is indeed a toxin, much less if it is involved in protection.

Case Studies

Toxins associated with obligate symbionts. While most insect inherited defensive symbionts that have thus far been described are facultative, two lineages, both of which employ toxins, stand out because they are obligate (i.e. the symbionts appear to be absolutely required for the host) – *Profftella* symbionts of psyllids, and *Streptomyces* symbionts of beewolves.

The β -proteobacterial symbiont *Profftella armatura* infects the Asian citrus psyllid *Diaphorina citri* (Hemiptera: Liviidae), a devastating pest of citrus that vectors the citrus

pathogen *Liberobacter asiaticus*, where it is housed in a symbiont organ (or bacteriome), along with another obligate symbiont, *Carsonella ruddi* (Nakabachi et al. 2013). As is common in many other sap-feeding Hemiptera, the two symbionts provide essential complementary vitamins and amino acids that are missing from the host's diet; *Proffittella* synthesizes riboflavin and biotin and *Carsonella* synthesizes histidine, tryptophan and other amino acids. Despite having a highly reduced genome, typical of obligate nutritional symbionts, 15% of *Proffittella*'s genome is composed of genes involved in the synthesis of a complex polyketide called diaphorin that is related to pederin made by symbionts of *Paederus* rove beetles. Phylogenetic analysis clearly indicates that the diaphorin synthesis genes were acquired via horizontal transfer. Although diaphorin is toxic to yeast and cultured mammalian and insect cells, it is not as toxic as pederin (Yamada et al. 2019). This may be because in diaphorin, the C10 methoxy group of pederin has been replaced by a hydroxyl group, which is thought to decrease hydrophobicity and binding to the ribosome (Mosey and Floreancig 2012). Laboratory studies show that diaphorin is also toxic to aphids and ladybird beetles when injected. However, it is not yet known which specific natural enemies diaphorin is effective against in nature. The Asian citrus psyllid has many natural enemies, including generalist predators (Michaud 2004) and more specialized parasitoid wasps (Chen and Stansly 2014). Since the related pederin toxin from rove beetles (discussed below) protects only against some groups of generalist predators, we might expect a similar protection against generalists by diaphorin. It would also be useful to determine whether *Proffittella* occurs in psyllids other than *D. citri*.

Solitary digger wasps in three genera in the subfamily Philanthinae (Hymenoptera: Crabronidae), commonly known as beewolves, are engaged in an amazing obligate defensive symbiosis with a lineage of *Streptomyces* bacteria. Adult females dig a burrow in the cell, where they lay a single egg and provision it with prey items. They also smear the burrow with *Streptomyces* that are housed inside specialized antennal glands. The wasp larva incorporates the bacteria into its cocoon, protecting it against generalist pathogenic fungi and bacteria in the soil. Experimental removal of bacteria resulted in over 90% larval mortality (Kaltenpoth et al. 2005), which explains why aposymbiotic wasps are rarely encountered. Upon emergence, the adult female wasp picks up bacteria from the cocoon, which colonize her antennal glands, continuing the cycle anew (Kaltenpoth et al. 2010). The symbiosis evolved once and has been stably maintained for ~70 million years. Although there is a strong degree of phylogenetic congruence

214 between hosts and symbionts, wasps have exchanged symbiont strains on a number of occasions,
215 possibly due to predation or reuse of nests (Kaltenpoth et al. 2014). Symbionts produce a
216 complex cocktail of antibiotics, consisting primarily of streptochlorin and piericidin derivatives,
217 and different compounds are effective against different pathogenic microbes (Engl et al. 2018,
218 Kroiss et al. 2010). Interestingly, the complement of antibiotics has remained rather stable over
219 the ~70 million years of the symbiosis.

220 *Toxins associated with facultative heritable symbionts.* The first report of symbiont-associated
221 toxins being incorporated into host defense involves staphylinid (rove) beetles in the genus
222 *Paederus* infected with the bacterium *Pseudomonas* (λ -proteobacteria). A factor contributing to
223 the early discovery of this defensive symbiosis is that paederine beetles have long been of
224 medical interest (possibly the cause of the biblical ‘plague of boils’) as painful lesions called
225 *Paederus* dermatitis develop when the hemolymph of some paederine beetles contacts human
226 skin. The blistering agent, named pederin, was identified as a polyketide amide in the 1960s
227 (Frank and Kanamitsu 1987, Gelmetti and Grimalt 1993). Pederin was shown to be toxic to a
228 range of cell lines and mice (Brega et al. 1968, Soldati et al. 1966). The presence of pederin in a
229 diurnal and brightly colored beetle (both atypical of rove beetles) suggested a potential role in
230 defense against vertebrate or invertebrate predators (Dettner 1987, Frank and Kanamitsu 1987).
231 Bioassays showed that pederin was not distasteful to vertebrate predators, nor did it reduce
232 feeding of insect predators relative to pederin-free beetles. However, it was found to deter
233 feeding by several species of wolf spiders and one jumping spider (Kellner and Dettner 1996).
234 While originally thought to be endogenously produced by the beetles, pederin was subsequently
235 linked to infection by an undescribed *Pseudomonas* species and the discovery of a polyketide
236 synthase (PKS) cluster in this bacterium confirmed the association (Kellner 2002, Piel 2002).
237 Polyketide synthases comprise a large protein family synthesizing a range of bioactive
238 compounds that are functionally diverse, including antifungal, antibacterial, anticancer, or
239 immunosuppressive properties (Staunton and Weissman 2001). Pederin belongs to the *trans*-AT
240 PKS group. Members of this group lack acyltransferase (AT) domains, although ATs are
241 encoded elsewhere in the polyketide synthase gene cluster and act *in trans* in an iterative fashion
242 (Piel 2009). The primary mode of action of pederin (and relatives -see below) is the inhibition of
243 protein synthesis leading to cytotoxic effects (Brega et al. 1968, Burres and Clement 1989,
244 Mosey and Floreancig 2012, Richter et al. 1997). Protein translation is disrupted by pederin’s

ability to bind to the E-site (final binding site for tRNA) of the large (60S) ribosome subunit resulting in the suppression of protein elongation (Dang et al. 2011, Nishimura et al. 2005). Other studies show that pederin also activates, possibly due to ribosome interference, protein kinase pathways, including p38 and JNK associated with stress-activated apoptosis (Lee et al. 2005).

In *Paederus*-associated *Pseudomonas*, the pederin synthase gene cluster is located on a horizontally transferred genomic island (Piel 2002). Pederin is structurally very similar to the theopederins and onnamides isolated from bacteria inhabiting marine sponges (Fisch et al. 2009, Piel et al. 2004), diaphorin from the Asian citrus psyllid ((Nakabachi et al. 2013) - see above), and nosperin from lichens (Kampa et al. 2013). This structural similarity, combined with phylogenetic analyses (see below), suggests these compounds have been shared among distantly related organisms via lateral gene transfer among host-switching symbiotic bacteria. Recently, non-symbiotic bacteria were also found to have pederin-like PKSs (e.g. (Kust et al. 2018)). Given that there are more than 600 species of *Paederus* beetles (and *Paederidus* beetles also bear this toxin), including 50 species known to cause contact dermatitis (Veraldi et al. 2013), PKS-based defenses may be widespread in paederine beetles although the protective effects of infection have only been documented for two species. Moreover, that PKSs are widespread in the genus *Pseudomonas* (which is itself an incredibly widespread microbe) and that pederin-like toxins appear in diverse host-associated bacteria, suggests PKS-mediated defense may be among the most widespread symbiont defenses in eukaryotes. While pederin-like toxins are associated with anti-predator phenotypes in terrestrial and marine systems, given the functional diversity of PKSs, as well as the taxonomic breadth of infected hosts, we may also expect to find symbiont-encoded polyketides that provide protection against parasites and pathogens.

Spiroplasma – *Spiroplasma* (Mollicutes) is an incredibly diverse, widespread, and likely non-monophyletic lineage of host-associated microbes (Anbutsu and Fukatsu 2011, Ballinger and Perlman 2019, Regassa and Gasparich 2006). *Spiroplasma* bacteria are found in a broad range of arthropods, including insects, arachnids, and crustaceans; a broad survey of western European terrestrial arthropods found that 7% (10/136 species) harbored *Spiroplasma* infections (Duron et al. 2008). Many *Spiroplasma* are gut commensals, others are pathogenic, including strains that cause disease in crabs, shrimp, and bees. Among the best-studied pathogenic *Spiroplasma* are leafhopper-vectored strains that cause disease in plants (Bove et al. 2003). Vertical transmission

has evolved independently numerous times in this genus (Haselkorn et al. 2009), including male-killing strains infecting butterflies, lacewings, beetles, aphids, planthoppers, and *Drosophila* flies ((Hayashi et al. 2016, Hurst et al. 1999, Jiggins et al. 2000, Poulson and Sakaguchi 1961, Sanada-Morimura et al. 2013, Simon et al. 2011). Although inherited *Spiroplasma* are transmitted primarily from mothers to their offspring over ecological timescales, phylogenetic analyses clearly demonstrate extensive horizontal transmission over evolutionary timescales (Haselkorn et al. 2009). The mechanism of horizontal transmission is not known. In *Drosophila* and aphids, inherited *Spiroplasma* are abundant in hemolymph and hemolymph transfusions are commonly used to establish infections in new host species (Haselkorn et al. 2013). Ectoparasitic mites have been shown to transfer *Spiroplasma* between fly hosts in the lab, and this may also be a common route of transfer in the wild (Jaenike et al. 2007).

Inherited *Spiroplasma* strains have been implicated in protection against diverse natural enemies in three different host lineages – *Drosophila* fruit flies, aphids, and tsetse flies. The mushroom-feeding woodland fly *Drosophila neotestacea* is commonly infected with a virulent generalist parasitic nematode, *Howardula aoronymphium*. Infection typically renders female *D. neotestacea* completely sterile. However, in flies that harbour *Spiroplasma* symbionts, fertility is fully restored, and nematodes appear sickly and small, and produce virtually no infective juveniles (Jaenike et al. 2010). Nematode infection is highly prevalent in the wild and the benefit provided by *Spiroplasma* to *D. neotestacea* is so great that *Spiroplasma*-infected flies are replacing their uninfected counterparts and rapidly spreading across N. America (Cockburn et al. 2013). In addition, *Spiroplasma* strains in *D. hydei*, *D. melanogaster*, and *D. neotestacea* have been shown to kill endoparasitic wasp larvae from two distantly related families – Braconidae and Figitidae (Haselkorn and Jaenike 2015, Xie et al. 2014, Xie et al. 2010). Inherited *Spiroplasma* have been reported in at least 18 *Drosophila* species, suggesting that defense may be common.

Toxins called ribosome-inactivating proteins (RIPs) have been implicated in *Spiroplasma* protection against both nematodes and wasps. RIPs are N-glycosidases that target a highly conserved adenine residue in the α -sarcin/ricin loop of eukaryotic 28S ribosomal RNA. They are especially widely distributed in plants and bacteria, and include the well-studied and deadly poisons Shiga toxin and ricin. The genomes of the *Spiroplasma* symbionts of *D. neotestacea* (sNeo) and *D. melanogaster* (sMel) respectively encode four and three secreted and

constitutively highly expressed RIPs (Ballinger et al. 2019). One of these RIPs was purified and shown to depurinate eukaryotic ribosomes (Hamilton et al. 2016). In addition, nematode and wasp ribosomes show characteristic signatures of RIP attack when parasitizing *Spiroplasma*-infected flies. Transfection experiments showed that sMel, unlike sNeo, does not provide protection (or show signatures of ribosome depurination) against nematodes (Ballinger et al. 2019, Haselkorn and Jaenike 2015). This may be because sNeo contains highly divergent RIPs, and different RIPs may be effective against different enemies. However, it is still completely unknown how *Spiroplasma* RIPs enter target cells. RIPs are typically classified into two types. Type II RIPs consist of an A unit that has glycosidase activity once inside the cell and a B unit that binds to a surface cell receptor, whereas Type I RIPs lack the A unit and are therefore considered less toxic. While *Spiroplasma* RIPs do not have a recognizable A unit, they have a long N-terminal region of unknown function. Transgenically expressing *Spiroplasma* RIPs inside *Drosophila* cells results in depurination of fly ribosomes (Garcia-Arraez et al. 2019), demonstrating that the glycosidase is effective against host ribosomes; host cells might, thus be resistant to toxin entry.

Genome sequencing has found that a wide range of *Spiroplasma*, including cultivable gut commensals, encode a diverse array of RIPs whose function is unknown (Ballinger and Perlman 2019, Hamilton et al. 2016). RIP evolution appears to be highly dynamic, and includes pseudogenized copies, as well as RIPs that are found on plasmids. The case of the European common red ant *Myrmica rubra* illustrates the dynamic nature of RIPs. Whereas ants in the native European and introduced North American range both harbor *Spiroplasma*, only symbionts of native ants appear to encode RIPs (Ballinger et al. 2018). The dynamic nature of RIPs suggests that they are gained and lost in conjunction with effectiveness against natural enemies.

Spiroplasma-mediated protection has also been demonstrated in aphids and tsetse flies. The mechanism of protection is not understood in either group, and aphid and tsetse *Spiroplasma* genomes have not yet been sequenced, so we do not know whether they harbour RIPs. A subset of *Spiroplasma* strains has been found to protect *A. pisum* pea aphids against the aphid fungal pathogen *Pandora neoaphidis* (Lukasik et al. 2013b) reducing overall aphid mortality as well as fungal sporulation. Transfection of pea aphid *Spiroplasma* into *Sitobion avenae* grain aphids also conferred protection (Lukasik et al. 2013a). A number of unrelated facultative symbionts, including *Regiella*, *Rickettsia* and *Rickettsiella*, also provided protection (Lukasik et al. 2013b),

with greater efficacy than most *Spiroplasma*, suggesting that defense is quite general. On the other hand, *Spiroplasma* strains do not appear to protect aphids against parasitic wasps. Mathé-Hubert and colleagues (2019) recently transfected 12 different *Spiroplasma* strains into the same pea aphid clonal genotype and tested their ability to protect against three strains of the parasitic wasp *Aphidius ervi*. They found that only one symbiont strain protected against the most virulent wasp strain. This strain was quite costly, suggesting that wasps were less successful because aphids were in poor health. Another recent study found that *A. ervi* wasps were less attracted to volatiles emitted by plants that had been fed on by *Spiroplasma*-infected aphids than by uninfected ones (Frago et al. 2017), suggesting an indirect way by which *Spiroplasma* might benefit aphids. The mechanism underlying this change in preference is unknown, but this effect was also observed in aphid harboring *Hamiltonella*, *Regiella*, *Serratia*, and *Rickettsia* facultative symbionts.

Finally, three species of *Glossina* tsetse fly were recently found to harbour *Spiroplasma* infections (Doudoumis et al. 2017). In controlled lab infections, *Spiroplasma*-positive flies were less likely to be infected with trypanosome gut parasites (Schneider et al. 2019); trypanosomatids were also more likely to be found in symbiont-free flies in the wild as well. The *Spiroplasma* in tsetse has very different dynamics and tissue tropism than in *Drosophila*, as it is found in the gut and not hemolymph, and at very high densities in larvae. More work is need to determine whether *Spiroplasma* infections result in reductions of gut parasites in tsetse, and if so, whether this effect is mediated by symbiont-encoded toxins.

Hamiltonella– Unlike the highly promiscuous *Spiroplasma*, another well-studied defensive symbiont, *Hamiltonella defensa*, appears restricted to hemipteran insects and is best studied in aphids (Moran et al. 2005b, Oliver et al. 2010, Oliver and Higashi 2019). Residing in the Yersiniaceae (order Enterobacteriales of the λ -proteobacteria), *Hamiltonella* forms a clade with two other common aphid facultative symbionts, *Regiella insecticola* and *Fukatsuia symbiotica* (Patel et al. 2019). Examined strains from this symbiont clade are all host-restricted, maternally-transmitted aerobic heterotrophs; although phylogenies indicate that occasional host switching occurs within and among species (Chevignon et al. 2018, Henry et al. 2013, Russell et al. 2003). In the lab, *Hamiltonella* can be readily moved among distinct aphid clones and species via microinjection, although not all genotypes are equally amenable to establishing stable infections (Lukasik et al. 2015, Niepoth et al. 2018, Oliver et al. 2005, Parker et al. 2017, Vorburger et al.

2010). The most likely natural routes of lateral transfer are wasp ovipositors (Gehrer and Vorburger 2012) and food plants (Li et al. 2018, Oliver et al. 2008), but the frequency of lateral transfer on ecological timescales (and hence its importance in symbiont maintenance in host populations) remains unknown. In aphids, *Hamiltonella* persists mostly in the hemolymph although it can establish in bacteriocytes and the surrounding sheath cells (Brandt et al. 2017, Moran et al. 2005b). Numerous published *Hamiltonella* genomes from pea aphids show that inventories of genes involved in housekeeping functions, nutrient acquisition, and metabolism are very similar among strains (and similar to other aphid facultative symbionts from the Yersiniaceae generally). In contrast, strains vary considerably in mobile DNA content, which comprises about ca. 25% of the typical *Hamiltonella* genome. This mobile DNA also results in large scale genome rearrangements among strains from distinct phylogenetic clades, while strains within the same clade exhibit similar architecture and mobile DNA content (Chevignon et al. 2018, Degnan et al. 2009).

Aphids have been developed as excellent models for studying phenotypic effects of infection with facultative symbionts, due to the ease of transfer among clonal lines, and the resulting abilities to ascribe symbiont-associated differences directly to impacts of the transferred symbionts (Brisson and Stern 2006, Oliver et al. 2010). Using such experimental approaches, *Hamiltonella* was first shown to protect the pea aphid, *Acyrtosiphon pisum*, from attack by its most common parasitoid, the aphidiine braconid wasp, *Aphidius ervi* (Oliver et al. 2003). While *Hamiltonella* infections were subsequently shown to modify aphid defensive and wasp oviposition behaviors (Dion et al. 2011a, Oliver et al. 2012), wasps nonetheless readily lay eggs in infected aphids, where *Hamiltonella* causes mortality to developing embryos or larvae, depending on symbiont strain; indicating a physiological basis of resistance (Martinez et al. 2014, Oliver et al. 2003). Later, *Hamiltonella* was shown to protect other aphid species from parasitoids, including *Aphis fabae* (black bean aphid), *Aphis craccivora* (cowpea aphid), *Rhopalosiphum padi* (bird-cherry-oat aphid) and possibly *Metopolophium dirhodum* (rose-grain aphid) (Asplen et al. 2014, Leybourne et al. 2020, Monticelli et al. 2019, Schmid et al. 2012, Vorburger 2014).

There is extensive strain variation in *Hamiltonella*-mediated defenses (Oliver and Higashi 2019). For example, a given strain often targets specific wasp species and different strains in an aphid species can confer variable levels of resistance against a single parasitoid species (Asplen et al.

2014, Cayetano and Vorburger 2013, Cayetano and Vorburger 2015, Hopper et al. 2018, Martinez et al. 2016, Martinez et al. 2014, McLean and Godfray 2015, Oliver et al. 2005, Schmid et al. 2012). Some studies have failed to find that particular *Hamiltonella* strains protect against one or more parasitoid species (Clarke et al. 2017, Łukasik et al. 2013). However, the aforementioned variation in target specificity suggests that symbiont defenses may simply be effective against other wasp species. Alternatively, in some systems *Hamiltonella* infections may be maintained in host populations by providing other benefits, including thermal tolerance, as suggested for pea aphids (Russell and Moran 2006), or influencing interactions with food plants as shown for whiteflies and grain aphids (Li et al. 2018, Su et al. 2015). Given that about one-third of the 5000 or so aphid species are estimated to carry *Hamiltonella* (Zytynska and Weisser 2016), aphid/*Hamiltonella* interactions may represent a wide-spread protective mutualism influencing interactions across a wide array of plant systems and their associated communities (McLean 2019, Oliver and Higashi 2019).

While *Hamiltonella*-based resistance to *A. ervi* was known to be physiological, based on experimental assays (Oliver et al. 2003), the first clues regarding the specific factors causing harm to wasps were derived from the first genomic studies. Most notably, partial sequencing of a moderately protective *Hamiltonella* strain identified a toxin-encoding bacteriophage, called APSE (for *Acyrtosiphon pisum* secondary endosymbiont), as a key factor of interest (van der Wilk et al. 1999; Moran et al. 2005a). In addition to the mere maintenance of an intact eukaryotic-toxin carrying temperate phage in a heritable symbiont, the case for APSE roles in defense were strengthened by variation in levels of protection correlating with specific APSE variants, in conjunction with APSEs occurring at high abundance relative to *Hamiltonella* and rt-qPCR assays showing that phage toxins were highly expressed (Martinez et al. 2014, Moran et al. 2005a, Oliver et al. 2005).

Subsequent experimental work confirmed that APSEs were required for the protection against parasitoids as phage loss resulted in the complete loss of the protective phenotype (Oliver et al. 2009), while re-introduction completely restored protection (Lynn-Bell et al. 2019). APSEs have other effects important in the regulation and maintenance of *Hamiltonella* (Weldon and Oliver 2016), including lowering the within aphid abundance of *Hamiltonella*, which impacts infection costs (Weldon et al. 2013). The ability to culture *Hamiltonella*, APSE and parasitoids *in vitro*,

established that *Hamiltonella* + APSE kills parasitoids without any aphid factors and confirmed *in vivo* findings that *Hamiltonella* alone is insufficient (Brandt et al. 2017). Mortality to wasps in conditioned media following the removal of *Hamiltonella*/APSE showed that a soluble factor is released from the symbionts that disrupts wasp development (Brandt et al. 2017). Furthermore, strains with published genomes that are virtually identical except for the presence of APSE confer either no protection (A2C, no phage) or nearly complete protection (AS3 with APSE) and the introduction of APSE *in vivo* or *in vitro* rapidly restores the protection (Brandt et al. 2017, Chevignon et al. 2018, Lynn-Bell et al. 2019).

APSEs are double-stranded DNA phages in the order Caudovirales with tail morphology similar to the Podoviridae and virion assembly genes showing homology to the P22 phage of *Salmonella enterica* (van der Wilk et al. 1999). Comparative genomics of APSEs (Rouil et al. 2020), however, show these phages exhibit mosaic genomes (36 – 40kb) composed of four modules that show high levels of synteny among modules and within the three modules associated with regulatory and structural function. The fourth module (2 - 7kb) contains virulence factors, including the toxins hypothesized to function in defense against parasitoids (Boyd et al. 2020, Chevignon et al. 2018, Degnan and Moran 2008a, Degnan and Moran 2008b, Dennis et al. 2017, Martinez et al. 2014, Moran et al. 2005a). APSE variants with distinct phage backbones, and occurring in *Hamiltonella* strains from different clades, can encode the same toxins. For example, the most common APSEs in North American pea aphids associated with alfalfa (*Medicago sativa*) are APSE2 and APSE8 (Weldon 2015). These each encode very similar homologs (99.3% nt similarity) of the active subunit (*cdtB*) of cytolethal distending toxin (CDT) (Chevignon et al. 2018, Moran et al. 2005a). Parasitism assays show that these *cdtB*-encoding APSE2 and APSE8 *Hamiltonella* confer similar and moderate levels of protection against *A. ervi* and no protection against the related aphidiine parasitoid *Praon pequodorum* despite the aforementioned variation in phage and bacterial strains (Doremus and Oliver 2017, Martinez et al. 2016, Martinez et al. 2014). Thus, along with phage loss and restoration studies, these correlations further support phage toxins as primary determinants of strength of resistance.

Cytolethal distending toxins are genotoxins associated with more than 30 proteobacterial and actinobacterial species, including the enteric pathogens *Yersinia*, *Escherichia* and *Shigella* that

are related to *Hamiltonella* (Jinadasa et al. 2011, Scuron et al. 2016). In most studied mammalian pathogens CDTs are AB₂-type heterotrimeric toxins comprised of the active subunit CdtB and the binding subunits CdtA and CdtC. The latter mediates toxin binding to the cell surface and delivery of CdtB into the targeted cell where it ultimately localizes to the nucleus; all three subunits are required for full activity (Jinadasa et al. 2011, Scott and Kaper 1994). The active subunit, CdtB, displays nuclease activity (homology to DNase 1), which arrests the cell cycle (usually in G2/M but in G1/S in some cell lines) leading to cell distention and death (Comayras et al. 1997, Lara-Tejero and Galan 2000, Pérès et al. 1997). Not all cell types are equally susceptible to CDT intoxication, which potentially explains why wasps are often killed by *Hamiltonella* with APSE2 or 8 with no apparent harm to aphids. More perplexing is how CdtBs exit *Hamiltonella* cells, then bind and enter wasp cells given that CdtA and CdtB are not present on the APSE or *Hamiltonella* chromosomes (Chevignon et al. 2018, Degnan and Moran 2008a, Degnan et al. 2009). CdtBs also occur in the *Hamiltonella* strains infecting black bean aphids (uncharacterized phage variant), *Chaitophorus* aphids (APSE6) and *Bemisia tabaci* whiteflies (APSE7) (Degnan and Moran 2008a, Dennis et al. 2017). They also occur in some strains of *Arsenophonus*, a widely distributed symbiont that frequently carries APSEs or phage elements related to APSE (Boyd et al. 2020, Duron 2014). It is unclear whether any *Arsenophonus* strains are protective, but in the red gum lerp psyllid, *Glycaspis brimblecombei*, there is a positive correlation between the prevalence of *Arsenophonus* infection and prevalence of attack by parasitic wasps (Hansen et al. 2007). CdtBs also occur on the main chromosome of *Fukatsuiia*, which lacks APSEs; these *cdtB* homologs are more similar to those occurring in *Yersinia* rather than those found in *Hamiltonella*-APSEs (Patel et al. 2019).

Recently, *cdtBs* were found in the genomes of several aphids and drosophilid flies (Verster et al. 2019). The presence in flies of *cdtBs* most closely related to those encoded by APSEs is especially surprising given that *Hamiltonella* has not been convincingly found outside of the Hemiptera. Perhaps some of these horizontal transfers were acquired from APSEs associated with *Arsenophonus* instead. It is not yet known whether the horizontally acquired *cdtB* genes function in defense as hypothesized (Verster et al. 2019), although their active sites are conserved, despite low overall protein sequence identity with their microbial homologs (Verster et al. 2019). In an interesting parallel, recent genome surveys have found that *Bemisia* whiteflies

and *Aedes* and *Culex* mosquitoes have acquired RIP toxin genes via horizontal transfer (Lapadula et al. 2017, 2019) although the donor was not *Spiroplasma* (see above).

The most protective strains of *Hamiltonella* characterized to date are those associated with APSE3, which encode a YD (tyrosine/aspartate)-repeat protein, a member of the rearrangement hotspot (RHS) family of toxins, in the phage virulence cassette. In pea aphids, APSE3-*Hamiltonella* confers complete (or nearly so) protection against the parasitoid *A. ervi*, but as with APSE 2 and 8, these strains show no activity against the related wasp *P. pequordoum* (Martinez et al. 2018, Martinez et al. 2016, Martinez et al. 2014, Oliver et al. 2009, Oliver et al. 2005). YDps also occur in the APSEs of protective *Hamiltonella* infecting black bean aphids (Dennis et al. 2017). Common in both gram-negative and gram-positive bacteria, RHS/YD-repeat proteins, including those in APSEs, are typically large, polymorphic exotoxins of 1,400 or more AA residues with the N-terminal core exhibiting YD repeats and the C-termini being highly variable, often with no homology to known effectors (Chevignon et al. 2018, Degnan and Moran 2008a, Jamet and Nassif 2015). Hence the specific mechanism(s) of action are not known. Related RHS/YD-repeat toxins are found in the mammal pathogen *Pseudomonas aeruginosa* (Kung et al. 2012), multiple strains of the nematode symbiont *Xenorhabdus* (e.g. (Kim et al. 2017)), the sulfur-oxidizing *Bathymodiolus* symbionts of hydrothermal vent mussels (Sayavedra et al. 2015), and *Vibrio* from Zebra fish (Stephens et al. 2015). They are also found in the entomopathogenic *Yersinia entomophaga*, which exhibit insecticidal activity (Busby et al. 2013). While many of these are thought to be defensive (Hillman and Goodrich-Blair 2016), Rhs/YD proteins from phylogenetically diverse bacterial lineages may be involved in bacterial kin recognition and mediate intracellular competition among bacteria via contact-dependent inhibition of growth (Koskiniemi et al. 2013). Aphids are frequently infected with more than one facultative symbiont, but symbiont distributions are non-random, with some combinations highly enriched and others depleted (Rock et al. 2018, Smith et al. 2015). While several factors may contribute to structure the heritable microbiome, microbe-microbe competition mediated by toxins such as YD are also potentially important in this regard (Doremus and Oliver 2017, Leclair et al. 2017, McLean et al. 2018, Oliver et al. 2006, Rock et al. 2018, Weldon et al. 2020).

The third toxin family associated with APSEs are Shiga toxins, a group of functionally related exotoxins that belong to the ribosome-inactivating protein (RIP) family discussed above with respect to *Spiroplasma*. Shiga toxins are also known from toxigenic *E. coli* strains (STEC, e.g.

serotype 0157:H7) and *Shigella dysenteriae*, which cause haemorrhagic colitis and haemolytic uraemic syndrome in humans (Johannes and Römer 2010). As with APSE-associated Shiga toxins, those from pathogenic bacteria are encoded by a range of bacteriophages, which through horizontal transmission contribute to genetic variation and confer novel traits that benefit the bacterial host (Herold et al. 2004). Lainhart and colleagues (2009) hypothesized that phage-encoded Shiga toxins in mammalian pathogens may have originally evolved in bacteria to protect against their eukaryotic predators. Their experiments show that the presence of the ciliate *Tetrahymena thermophila* induces toxin production, which kills the predator. The Shiga holotoxin has an AB₅ molecular configuration, in which the active subunit StxA binds to five identical fragments that form StxB. The StxB subunit binds to glycolipid receptors (globotriaosylceramides) resulting in tubular invaginations critical to uptake into host cells. As with other RIPs, once inside the cell, StxA inhibits protein synthesis by cleaving a specific adenine base of 28S rRNA, preventing amino-acyl tRNA binding and hence chain elongation (Endo et al. 1988, Johannes and Römer 2010, Saxena et al. 1989). In several APSE variants (1, 4 and 5) it has been found that the P7 protein shows weak homology to StxB (Degnan and Moran 2008a, Dykstra et al. 2014, van der Wilk et al. 1999), while P9 has been hypothesized to be the functional analog of StxA.

The protective phenotype of Stx-encoding APSE5 from *Hamiltonella* infecting *Uroleucon rudbeckiae* has not been characterized, but a *Hamiltonella* strain APSE1 from pea aphids confers high levels of protection against the braconid *A. ervi*, and low levels against the chalcid wasp *Aphelinus abdominalis* (McLean and Godfray 2015). Also APSE4/*Hamiltonella* infecting the cowpea aphid, *Aphis craccivora* conferred high levels of protection against two *Binodoxys* species, but not two other braconid parasitoids (Asplen et al. 2014, Dykstra et al. 2014). In APSE4, the *stxB* homolog (P7) is pseudogenized by an inactivating insertion, raising questions as to the importance of the putative delivery subunit in this protective mutualism. In the aforementioned study where Shiga toxins protect their bacterial hosts against protist predators, disruption of StxB substantially reduced StxA activity (Lainhart et al. 2009).

An emerging topic in symbiosis research is to ask how changing temperature affects the stability and outcome of symbiosis (Corbin et al. 2017); this is especially pertinent with global climate change. Interestingly, *Hamiltonella* protection fails at higher temperatures (Doremus et al. 2018, Higashi et al. 2020) and this may be partially or wholly mediated by defensive toxin thermal

sensitivity. CdtBs are well-known to be heat labile (Johnson and Lior 1988) and *cdtB*-encoding APSE2 and APSE8 strains completely fail at only moderately warmer temperatures (Doremus et al. 2018). YD-encoding APSE3 strains only partially fail under exposure to warmer temperatures, but little is known about how YD proteins are affected by heat. Shiga toxins do not appear heat labile (Rasooly and Do 2010), but APSE do not encode homologs of StxA and it is unclear whether the putative P9 homolog is heat labile; also no parasitism assays to date indicate whether these strains also fail at higher temperatures.

The strains of *Hamiltonella* infecting whiteflies form a distinct clade, are restricted to bacteriocytes, and the APSE phages appear inactivated (Chevignon et al. 2018, Gottlieb et al. 2008, Rollat-Farnier et al. 2015). It is likely then, that *Hamiltonella* is not generally serving as a defensive symbiont in whiteflies, although a recent study found a correlation between *Hamiltonella* frequency and parasitism (Qi et al. 2019). There are reports that whitefly *Hamiltonella* can influence the transmission of plant pathogenic viruses (Gottlieb et al. 2010), impact sex ratios (Shan et al. 2019), and affect plant signalling to suppress induced defenses (Su et al. 2015), a finding recently reported in aphids as well (Li et al. 2019).

Aphids host two other defensive facultative symbionts that are close relatives of *Hamiltonella* – *Regiella insecticola* and *Fukatsuia symbiotica*. *Regiella insecticola* was the first aphid symbiont shown to provide defense against the specialized fungal pathogens *Pandora neoaphidis* (Order Entomophthorales) (Scarborough et al. 2005) and *Zoophthora occidentalis*, but not the generalist pathogen *Beauveria bassiana* (Parker et al. 2013). Subsequently, most other common aphid facultative symbionts, even distantly related species, were shown to protect against *Pandora*, including *Rickettsia* (α -proteobacteria), *Spiroplasma* (Mollicutes), and a range of γ -proteobacteria: *Fukatsuia*, *Rickettsiella* and possibly *Hamiltonella* (Heyworth and Ferrari 2015, Lukasik et al. 2013b, Weldon et al. 2020). The mechanism of symbiont-based resistance to fungal pathogens is unknown. But given that almost all aphid facultative symbionts confer this trait, a more general response, such as immune priming, may underlie this form of symbiont-encoded protection. A strain of *Regiella* from *Myzus persicae* (green peach aphid) was also found to confer protection against the aphidiine parasitoid, *Aphidius colemani*, in its native host and when transferred to black bean aphids (Vorburger et al. 2010). The same strain also conferred resistance against *A. ervi* when transferred into pea aphids (Hansen et al. 2012, Vorburger et al. 2010), and a naturally occurring pea aphid strain conferred protection against

this same parasitoid, but only in some aphid genotypes (Oliver et al. 2003, Weldon et al. 2020). *Regiella* does not contain APSE phages, and while the genome of the protective *Regiella* strain from *Myzus* was found to encode additional toxins and pathogenicity factors not found in a ‘non-protective’ strain from pea aphids, it remains unclear how *Regiella* harms parasitoids (Degnan et al. 2010, Hansen et al. 2012). The ‘non-protective’ *Regiella* strain from the Hansen et al. (2012) study appears similar to a strain that did provide protection against the same parasitoid depending on aphid genotypes (Weldon et al. 2020).

Forming a clade of facultative symbionts with *Regiella* and *Hamiltonella*, the symbiont *Fukatsuia symbiotica* has been reported to provide the full suite of defensive services seen across all pea aphid facultative symbionts (against parasitoids, fungal pathogens or thermal stress) (Heyworth and Ferrari 2015). Another study found that the dominant North American strain infecting alfalfa-associated pea aphids provided none of these benefits. Instead, the authors hypothesized that *Fukatsuia* exploits the *Hamiltonella* protective mutualism and spreads within pea aphid populations via hitchhiking (Doremus and Oliver 2017, Doremus et al. 2018). In Lachninae aphids, *Fukatsuia* is thought to provision B vitamins, as their *Buchnera* obligate symbionts have lost this ability (Meseguer et al. 2017, Russell et al. 2017). As with *Regiella*, *Fukatsuia* also does not contain APSEs but as noted above does have an intact *cdtB* homolog encoded on the main chromosome. This *cdtB* carrying strain, however, provided no protection against the wasp *A. ervi*. All three of the symbionts in this clade encode a range of overlapping pathogenicity factors, including a large number of repeats in toxin (RTX) (Patel et al. 2019). Named after the repeating glycine and aspartate repeats at the C-terminus, RTX toxins are associated with a variety of gram-negative bacteria with protein translocation across the bacterial membranes typically provided via Type-I secretion systems (T1SS) (Linhartová et al. 2010). While some RTXs are associated with cytotoxic activity, including pore-forming toxins, others are involved with a variety of biological activities (e.g. multifunctional autoprocessing RTX or MARTX toxins). It is unclear if RTX, or the other common pathogenicity factors encoded in these genomes, aid in protective phenotypes, either directly or indirectly.

Lagria symbionts – The most recent addition to the list of inherited toxin-producing defensive symbionts of insects is found in tenebrionid beetles in the subfamily Lagriinae. All species

examined thus far (in the genera *Lagri*a and *Ecnolagri*a) harbor extracellular *Burkholderia* bacteria that are housed in adult female accessory glands in the reproductive tract, and that are vertically transmitted to offspring (Florez and Kaltenpoth 2017). In lab assays, the bacteria were found to protect eggs and larvae from pathogenic fungi (Florez et al. 2017). Although both male and female larvae host the symbiont, adult males are not infected. Also, 80-90% of adult females are infected in the wild, suggesting that although the infection is highly prevalent, it is not obligate. Multiple strains of *Burkholderia* are involved in the symbiosis, and individual beetles can harbor more than one oligotype. Although these strains are all closely related to *B. gladioli*, there are interesting differences. For example, the most abundant strain in the field is relatively rare in beetles that have been maintained in the lab. It is as yet uncultivable and has a reduced genome that encodes toxic compounds that are not found in other lagriine *Burkholderia* symbionts, such as lagriamide, a novel polyketide that is similar to poisons found in ascidians (and suspected to be synthesized by ascidian bacterial symbionts) and whose biosynthetic gene cluster has been acquired by horizontal gene transfer (Florez et al. 2018; Waterworth et al. 2019). On the other hand, the most abundant strain in lab-reared beetles is rare in the field. It can be cultured and produces a wide range of poisons, including polyketides, aromatic glycosides, and lipopeptides, some of which exhibit activity against bacteria or fungi in lab assays (Dose et al. 2018, Florez et al. 2018). There is thus a rich pool of defensive *Burkholderia*, with a diverse repertoire of antimicrobial compounds. Although at least some lagriine *Burkholderia* are vertically transmitted, phylogenetic analysis demonstrates that there is much horizontal transmission. Interestingly, *B. gladioli* is a common plant pathogen and the most common cultivable lagriine *Burkholderia* was shown to be able to be transmitted from beetles to soybeans, where they induced an immune response and caused a reduction in seed production (Florez and Kaltenpoth 2017). The diversity of lagriine *Burkholderia* and the current inability to culture the most abundant field strain makes it challenging to understand whether and how *Burkholderia* strains mediate interactions between beetles and their host plants.

Three major questions in toxin-mediated defensive symbiosis

In this section, we highlight three major unresolved questions in the field.

1. From where do endosymbiont-encoded toxins originate?

As noted above, many of the toxins functioning in characterized defensive symbioses have wide distributions in bacteria, often facilitated by their localization on phages, plasmids and other mobile elements. For example, the toxins found in *Hamiltonella*, have homologs in diverse bacteria. In *Hamiltonella*, these toxins occur on temperate phages (APSEs), which possibly moved from the widespread facultative symbiont *Arsenophonus* into seemingly hemipteran-restricted symbionts (Boyd et al. 2020, Duron 2014). More broadly, ds-DNA tailed bacteriophages have mosaic genomes comprised of modules with distinct evolutionary histories. These modules typically contain sets of genes that function together, often encoding completely different proteins (Casjens and Thuman-Commike 2011). APSEs, for example, contain a module with homology to a nonintegrative phage ϕ SG1 infecting *Sodalis*, a symbiont of tsetse flies (Clark et al. 2007, Degnan and Moran 2008a). Thus, toxins may be shuffled within and among bacteriophages, which in turn infect variable ranges of symbiont hosts. This modular evolution of phages may create novel toxins. As noted above, several APSEs encode homologs of StxB, important for toxin delivery into target cells, but not the active toxin StxA; instead, a hypothesized analog to StxA is present (Degnan and Moran 2008a). Coinfections of facultative symbionts are common in insects (Ferrari et al. 2012, Jing et al. 2014, Rock et al. 2018, Russell et al. 2013, Smith et al. 2015, Toju and Fukatsu 2011, Zchori-Fein et al. 2014, Zytynska and Weisser 2016), providing ample ecological opportunities for mobile-element mediated toxin transfers. Recombinant phage with new toxins may not be beneficial to bacterial or animal hosts, or may impose costs, resulting in their inactivation and loss. Virtually all defensive symbionts arise from lineages with free-living or host-restricted pathogens as near ancestors. Hence many pathogenicity factors, including some toxins, were likely present on chromosomes at the initiation of the symbiosis. For example, while their function has not been characterized, multiple, highly similar copies of RTX occur in *Fukatsuia*, *Hamiltonella* and *Regiella* indicating they were likely present in their common ancestor with some diversification occurring after the split (Patel et al. 2019). However, it is important to note that all of the primary toxins thought to be involved in host defense occur on mobile elements, and some (e.g. PKS) are shared broadly across the animal world.

2. Do symbiont toxins harm hosts? & 3. How do natural enemies fight back/protect themselves against symbiont-mediated toxins?

675 Defensive symbiont toxins also have the potential to harm the host of the defensive symbiont.
 676 While defensive symbionts clearly provide conditional benefits that allow for their spread and
 677 persistence in host populations, infections can also potentially incur both constitutive and
 678 induced costs (Cockburn et al. 2013, Jaenike et al. 2010, Kach et al. 2018, Martinez et al. 2018,
 679 Oliver et al. 2008, Smith et al. 2015, Vorburger and Gouskov 2011). Many defensive symbionts
 680 lack genes for the biosynthesis of essential amino acids and other nutrients, yet retain transporter
 681 genes for their import. This indicates that they are nutritional parasites on some level
 682 contributing constitutive costs connected to maintaining infections (Ankrah et al. 2017,
 683 Chevignon et al. 2018, Patel et al. 2019). Of course, repeated interactions with the host immune
 684 system may also contribute to constitutive costs as antimicrobial peptides or other factors may be
 685 continuously deployed, in part to limit the distribution and/or proliferation of symbionts (Login
 686 et al. 2011, Ratzka et al. 2012). Costs of deploying toxins in host defence may also result in
 687 induced costs as the toxins that harm enemy tissues may also harm host tissues. While induced
 688 costs have not been reported in insect defensive symbionts (Vorburger et al. 2013) these costs are
 689 harder to measure as the enemy-challenge itself introduces costs that are difficult to disentangle
 690 from induced costs. Little is known about toxin delivery mechanisms in defensive symbionts,
 691 which are likely to impact induced costs. For example, in aphids, parasitism may induce the lysis
 692 of *H. defensa*, which may release toxins into the hemocoel and expose aphid tissues to these
 693 toxins (Martinez et al. 2014). However, if other factors deliver and insert toxins into target cells,
 694 or symbionts invade target cells and then lyse, then induced costs may be minimal. Transgenic
 695 *Drosophila melanogaster* engineered to express a *Spiroplasma* ribosome-inactivating protein
 696 showed reduced fitness, in terms of reduced adult survival and elevated embryonic mortality
 697 (Garcia-Arraez et al. 2019), but it is hard to determine how much this is due to toxins that remain
 698 inside the fly cell and damage fly ribosomes, as opposed to secretion and re-entry.

699 Some defensive toxins, including *cdtB*, lack the binding subunits required for delivery, but other
 700 factors may have replaced these functions due to the modular nature of toxin evolution. As a
 701 reminder, many defensive symbionts exhibit high target specificity. Pederin in rove beetles
 702 harmed spiders but not insect predators (Kellner and Dettner 1996), and *H. defensa* strains are
 703 often effective only against particular parasitoid species, even closely related ones (Asplen et al.
 704 2014, Cayetano and Vorburger 2015, Martinez et al. 2016, McLean and Godfray 2015). This

could indicate that the defensive symbiont toxins or delivery mechanisms are highly specific, and so would be less likely to harm unrelated hosts.

Parasitoids can rapidly evolve resistance to defensive symbionts, and this is hypothesized to be mediated by toxins in *H. defensa*/APSE-infected aphids (Dennis et al. 2017, Dion et al. 2011b, Rouchet and Vorburger 2014). More generally, co-evolutionary interactions may maintain resistance-conferring symbiont and toxin diversity, and counter-resistance mechanisms in parasitoids (Hafer and Vorburger 2019, Vorburger and Perlman 2018). Target specificity may also mediate competition between rival parasitoids (Kraft et al. 2017, McLean and Godfray 2017), which can select for or against specific defensive toxins.

Parasite counter-resistance is one of the least-studied aspects of defensive symbiosis, and also one of the most promising in terms of disentangling underlying mechanisms of protection. As mentioned above, the best examples of both fine-scale specificity and enemy counter-resistance come from aphid symbionts, with studies demonstrating effective protection against some genera but not others (e.g. (Asplen et al. 2014, Martinez et al. 2016)), all the way to specificity at the level of host, symbiont, and wasp genotype (Rouchet and Vorburger 2012). Wasps can rapidly evolve resistance to symbiont-mediated protection (Rouchet and Vorburger 2014), for example by superparasitism (Oliver et al. 2012) or avoiding symbiont-infected aphids (Łukasik et al. 2013). Mateos and colleagues (Mateos et al. 2016) recently found three *Drosophila*-parasitic wasp species that were able to overcome *Spiroplasma* protection. These resistant wasps are all close relatives of susceptible species, which presents a promising opportunity to study mechanism of protection and counter-resistance in this system.

Another way to study enemy counter-resistance is to examine the evolutionary persistence of symbiosis and defense. For example, philanthine beewolves have hosted *Streptomyces* symbionts with a conserved toxin repertoire for ~70 million years, which shows that there has been little resistance evolution in this system. Resistance may be difficult to evolve here because there is a complex cocktail of poisons to detoxify and because the target microbes are opportunistic pathogens living in the soil, with therefore weaker selection for resistance than a developing parasitoid for example. Thus, we can contrast this stability with *Hamiltonella* and *Spiroplasma* defensive symbioses, which are highly dynamic, with symbionts and/or symbiont toxins being

rapidly gained and lost, presumably due to the effectiveness of the toxin and the cost/benefit of carrying it.

For phage-encoded toxins, their production has the potential to mediate competition between bacterial cells with and without phage. This can have implications for protective function and the regulation of the defensive symbiosis. For example, within an individual aphid, not all *Hamiltonella* cells are infected with APSEs (Degnan and Moran 2008a). If toxin production or other mechanisms result in phage infected cells suffering costs relative to uninfected cells, then within-host selection has the potential to reduce the frequency of APSE-carrying *Hamiltonella*, and phage losses may become fixed through vertical transmission bottlenecks. For APSE3 *Hamiltonella*, spontaneous phage losses occur repeatedly, while APSE2/8 associated *Hamiltonella* appear stable (Oliver et al. 2009). Phage loss results in complete loss of protection, but also increases in *Hamiltonella* abundance, which correlates with severe fitness costs to aphids where host-level selection is expected to remove them from the population (Oliver et al. 2009, Weldon et al. 2013). As with some pathogens (e.g. (Sturm et al. 2011)), toxin production may also be metabolically costly, which could also select for phage-free bacteria or add to constitutive infection costs.

In pathogens, in addition to performing ‘offensive’ roles in harming host tissue, toxins may also serve defensive functions by compromising the immunity of the bacterium’s host, or even benefit bacteria in ways unrelated to these direct interactions, including roles in motility and biofilm formation (Rudkin et al. 2017). The defensive toxins identified in insect symbionts may similarly perform roles beyond direct toxicity. While such roles are unknown at present, and difficult to study, some common putative toxins in defensive symbionts, including RTX toxins, are already associated with diverse functions in other bacteria (Linhartová et al. 2010). Defensive symbionts have reduced genomes compared to their free-living relatives, yet about a quarter of their genomes is comprised of mobile DNA (Chevignon et al. 2018, Degnan et al. 2010, Degnan et al. 2009, Patel et al. 2019). Pathogenicity islands derived from acquired phages, plasmids or transposons, contain diverse toxins, adhesins, secretion systems, and iron uptake systems, which can have roles in pathogenicity and/or symbiosis (Hacker and Kaper 2000, Patel et al. 2019). Hence, many facultative symbiont genomes are full of putative toxins and other pathogenicity factors. Yet it is entirely unclear what these do, and whether they play a role in protective symbiosis or provide other benefits to the bacterial or insect host. Long term host-restriction in

heritable symbiosis combined with reduced opportunities for horizontal exchange with other bacteria, might presumably lead to selection for attenuated virulence and their removal - so why are they retained? Are they providing functions in service of the defensive symbiosis, such as roles required for persistence and vertical transmission in their hosts? This would involve navigating interactions with the insect immune system, reaching and occupying vital tissue for transmission, or resolving competition with other microbes. Alternatively, the bulk of these may be maintained by selfish genetic elements that prevent their removal. Toxin-antitoxin systems (aka ‘addition modules’) can enable plasmid persistence by eliminating cells that did not inherit a copy, but when present on the main chromosome they may ‘stabilize’ genomic islands by preventing large deletions (Van Melder and Saavedra De Bast 2009). Other factors, including toxin-antidote bacteriocins may perform similar functions (Inglis et al. 2013).

Conclusion

It is a truly exciting time to study defensive symbionts of insects. Genomic and proteomic approaches are allowing us to not only identify novel toxic factors, but to also examine population-level variation in protective ability. Advances in experimental approaches, such as the ability to culture and genetically transform symbionts (Brandt et al. 2017, Masson et al. 2018), or to express symbiont genes in model organisms amenable to transgenics (Beckmann et al. 2017, Harumoto and Lemaitre 2018, LePage et al. 2017), will allow us to probe function in exciting detail. And of course, we have only reached the tip of the tip of the iceberg in discovering new defensive symbioses in the wild.

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Table 1. Summary of the best characterized inherited defensive symbionts in which toxins have been implicated in protection

Symbiont	Insect host	Distribution & Prevalence	Mode of transmission	Toxin	Natural enemy targeted by toxin
<i>Burkholderia</i>	Lagriine beetles (Coleoptera: Tenebrionidae)	Found in 2 genera; 80-90% of adult females are infected	Extracellular, housed in female accessory glands, and deposited over the surface of the egg	Different strains produce diverse polyketides, aromatic glycosides, and lipopeptides	Fungi & bacteria
<i>Hamiltonella</i>	Aphids (Hemiptera: Aphididae)	Widely distributed in aphids with variable prevalence among species; protection has been demonstrated in 5 species	Intracellular, in embryos and eggs	Phage encoded YD repeat, CdtB, and Shiga-like toxins	Parasitic wasps
<i>Profftella</i>	<i>Diaphorina citri</i> - Asian citrus psyllid (Hemiptera: Liviidae)	Only known thus far from 1 species, where it infects all individuals (obligate)	Intracellular, bacteriome-restricted	Diaphorin polyketide	Unknown
<i>Pseudomonas</i>	Paederine beetles (Coleoptera: Staphylinidae)	Widely distributed across paederines, but within species, at least some individuals are uninfected	Females deposit bacteria over the surface of the egg; larvae can acquire and transmit symbionts by eating eggs	Pederin polyketide	Predatory spiders
<i>Spiroplasma</i>	<i>Drosophila</i> flies (Diptera: Drosophilidae)	~20 infected species, with prevalence ranging widely, from ~4-85%; protection has been demonstrated in 3 species	Inside egg	Ribosome-inactivating protein	Parasitic wasps, parasitic nematodes
<i>Streptomyces</i>	Philanthine beewolves (Hymenoptera: Crabronidae)	Found in 3 genera; virtually all females are infected (obligate)	Housed in adult female antennal glands and smeared in offspring's burrow, picked up by emerging adult female to continue the cycle	Cocktail of streptochlorin and piericidin derivatives	Fungi

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