CHAPTER NINE

The saboteur's tools: Common mechanistic themes across manipulative symbioses

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Abstract

Insects and other arthropods often harbour intracellular bacterial associates. These bacterial symbionts cannot survive outside their host and rely on vertical transmission from infected mothers to their progeny. Thus, symbiont success is tied directly to reproductive success of female hosts. As a result of this intimate relationship, these heritable symbionts have evolved numerous strategies to increase the likelihood of their own transmission, some of which involve the direct manipulation of host reproduction to increase production or fitness of female progeny. These manipulations often come at the expense of male hosts or uninfected individuals, and include strategies such as inducing crossing incompatibilities that increase infected female fitness relative to uninfected females (cytoplasmic incompatibility), killing or feminizing male hosts, and inducing asexual production of female progeny (parthenogenesis). In the past decade, there has been substantial interest in the applied use of these manipulative bacteria in control of pest species, including suppressing disease carried by mosquito vectors of human pathogens. This, along with developments in molecular tools and techniques, has spurred major advances in understanding the mechanisms by which symbiont lineages manipulate their insect hosts, culminating with the identification

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of the bacterial genes responsible for key manipulations. Here, we review the major research advances in the mechanisms of symbiont-induced reproductive manipulation and compare the mechanisms of a number of common reproductive manipulators.

1. Introduction

Symbioses between insects and bacteria that live within them intracellularly or within bodily fluids are a "pandemic" (Duron et al., 2008; Werren et al., 2008); most insects bear these intimate associates. The bacteria in these partnerships are almost exclusively transmitted vertically from infected mothers to their progeny and are unable to survive outside of their host. To propagate, these heritable bacteria rely on the survival and successful reproduction of infected females. The intertwined fitness of host and symbiont has spurred the development of numerous strategies used by symbionts that increase the likelihood of their transmission. These include direct manipulation of the host's reproduction in a way that increases the number or fitness of female hosts, usually at the expense of male or uninfected hosts. These "reproductive manipulators" infect an estimated 40%–50% of all insects, as well as other arthropod lineages like crustaceans and chelicerates (Weinert et al., 2015; Zug and Hammerstein, 2012).

To date, four main types of reproductive manipulations have been identified from a diverse array of symbionts, representing many of the major bacterial phyla and even some microbial eukaryotes. The most common manipulation, cytoplasmic incompatibility (CI), is a crossing incompatibility that, at its simplest, results in offspring mortality in crosses between infected males and uninfected females. Bidirectional CI can also occur when both partners are infected with different symbiont strains (Werren et al., 2008). Mortality is averted when the female harbours the same symbiont strain, with bacteria or bacterial products in the infected cytoplasm "rescuing" the embryo. Another manipulative strategy is male-killing, in which infected males die at some point during development. A third is feminization, which occurs when genetically male hosts develop as functional females, and a fourth is parthenogenesis induction (PI), in which infected, mostly haplodiploid, hosts asexually produce infected daughters. While these phenotypes have historically been considered separately, there is increasing evidence that suggests that symbionts inducing different phenotypes may target the same host processes, or that symbionts causing one

phenotype may contain the genetic capability to induce other manipulations (Fig. 1). For example, the successful transinfection of novel host species with well-characterized symbiont strains has sometimes revealed unexpected switches in manipulative phenotypes, and recent analyses of the genetic repertoire of these bacterial manipulators have revealed that many harbour the genes to induce multiple types of manipulations.

Despite their importance, the dependence of heritable symbionts on the host environment and our inability to culture them has made their study challenging. Typical microbiological methods, including direct manipulation of gene expression are not available. Although identified in numerous insect lineages as early as the 1920s, research on these bacteria was a daunting task until the development of molecular techniques like PCR. The difficulty of manipulating these symbionts has made the mechanisms by which control of host reproduction is achieved a long-standing mystery. However, by largely observational methods that take advantage of well-studied genetic host model systems, as well as various "omics" approaches, recent research on manipulative symbionts has made large strides towards elucidating the mechanisms by which these bacteria modify their arthropod hosts.

Here, we first review the current knowledge of the mechanisms controlling a variety of phenotypes conferred by heritable symbionts. In so doing, we hope to draw attention to those symbionts and mechanisms that are comparatively less well-characterized and compare potentially similar mechanisms of distantly related symbionts. We also discuss the mechanistic implications of the shifting phenotypes observed in novel manipulative infections, and the similarities seen across the four manipulative strategies. In doing so, we highlight common mechanistic themes of various arthropod-associated manipulative symbionts.

2. Cytoplasmic incompatibility (CI)

Cytoplasmic incompatibility is the most widely studied of the four reproductive manipulations, in part because of its ubiquity, and its occurrence in the model insect *Drosophila melanogaster*. CI also has long been of interest because of its potential to suppress pest populations via release of infected males (Laven, 1967). More recently, the discovery that some strains of the most common CI-inducing symbiont, *Wolbachia*, increase mosquito resistance to human vector-borne pathogens (Frentiu et al., 2014; Moreira et al., 2009; Walker et al., 2011) has refocused the community on understanding the mechanisms behind the CI and pathogen-blocking phenotypes

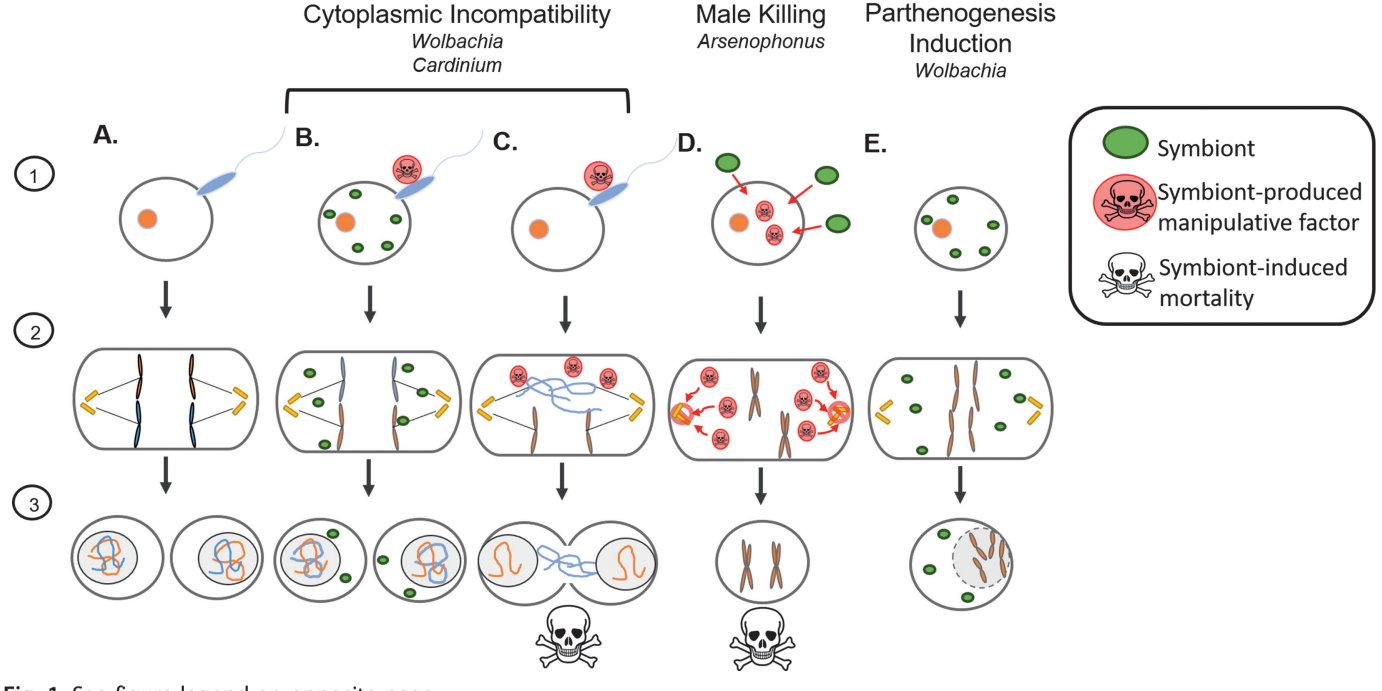

Fig. 1 See figure legend on opposite page.

(Caragata et al., 2013; Caragata et al., 2019; Rances et al., 2012; Thomas et al., 2018). This conferred resistance to pathogens, combined with the genetic drive capabilities of CI has spurred substantial additional interest in the study of CI-causing symbionts and has led to releases of *Wolbachia*-infected mosquitos in regions with dengue virus activity (Hoffmann et al., 2011).

Early crossing experiments of mosquitoes infected with different *Wolbachia* strains revealed the potential for complex crossing types between different strains, with some strains either rescuing or failing to rescue the CI of other strains, and even *Wolbachia* strains that could only rescue, but not induce CI (Atyame et al., 2011; Bourtzis et al., 1998; Charlat et al., 2004; Laven, 1967). The complexity of these crossing types conformed to a verbal model of "modification" of sperm in males and "rescue" by cytoplasm of infected females (Werren, 1997), and suggested that the CI modification/rescue process of CI must involve at least two genes: a modification and a rescue factor. The modification and rescue system is conceptually similar to bacterial toxin/antitoxin systems; in these systems, the short-lived antidote and long-lived toxin typically form an operon and are expressed together. The antidote prevents toxicity when present, often by directly

Fig. 1 Symbiont-induced reproductive manipulations that directly target the eukaryotic cell cycle. (A) Early stages of normal embryonic development including the first round of mitosis. (1) Fertilization of uninfected egg, (2) normal anaphase, with sister chromatids both condensed and fully separated, (3) completion of mitosis and the cell cycle culminating in two daughter cells. (B) Rescue of symbiont-induced cytoplasmic incompatibility beginning with (1) fertilization of CI-symbiont-infected egg cell with CI-modified sperm, (2) presence of CI-symbiont throughout mitosis promotes successful chromatid condensation and separation, (3) successful CI rescue and cell replication. (C) Cellular defects associated with CI mortality. (1) Fertilization of uninfected egg cell with CI-modified sperm, (2) Modification factor affects paternal chromatin condensation, (3) cell attempts to complete cell replication, resulting in paternal chromatin "bridging" between the two daughter cells prior to embryo mortality. (D) *Arsenophonus*-associated male-killing. (1) *Arsenophonus* male-killing factor diffuses into egg cell, (2) male-killing factor targets maternally-derived centrosomes that form in unfertilized male eggs, (3) centrosomes do not produce a mitotic spindle and the cell fails to divide, leading to male-specific embryonic mortality. (E) Gamete duplication variant of parthenogenesis induction. (1) Unfertilized egg infected with PI symbiont, (2) after DNA replication, sister chromatids do not fully separate during early embryonic mitosis, (3) daughter nuclei fuse back together, resulting in a diploid cell. Green circles represent the symbiont manipulator, circle skull represents a symbiont factor involved in manipulation. **Wolbachia* and *Spiroplasma* use a different mechanism(s) to kill males by targeting the dosage compensation system. For brevity, only *Arsenophonus* that specifically targets cell cycle-associated organelles is shown. *Cardinium* and *Rickettsia* PI target meiosis, where the reduction division is aborted, not mitosis as depicted here.

binding the toxin. However, the antidote must be continuously produced in order for continuous blocking of the toxin. Failure to produce the antidote, typically through the loss of the extrachromosomal element harbouring the toxin-antidote operon, results in exposure to the longer-lived toxin and results in the mortality of the host organism (Van Melderen and Saavedra De Bast, 2009).

After nearly a century since the first discovery of *Wolbachia* in mosquitoes, bacterial genes responsible for CI have been identified. A protein associated with the *Wolbachia*-infecting bacteriophage, WO, was identified in the spermathecae of uninfected female mosquitos after mating with infected males harbouring the *w*Pip strain (Beckmann and Fallon, 2013; Bordenstein and Bordenstein, 2016). This protein was named *cidA* and was found to be directly upstream of a second gene that harbours a deubiquitinase domain, *cidB*, suggesting that these two genes could form a CI factor operon, with *cidA* acting as the hypothetical rescue (or antidote) factor and *cidB* acting as the modification (toxin) factor (Beckmann et al., 2017; Beckmann et al., 2019). Based on other toxin-antidote systems found in free-living bacteria, it has been hypothesized that the genes for the CI factors also form an operon (Beckmann et al., 2017; Beckmann et al., 2019; Van Melderen and Saavedra De Bast, 2009). In further support of their status as a toxin-antidote system, *cidA* and *cidB* proteins show high binding affinity for one another (Beckmann et al., 2017). However, the operon status of these loci has been debated, given that the *cid* genes exhibit quite different expression levels (Lindsey et al., 2018). While operon-associated genes typically show similar expression levels, this is not always the case (Güell et al., 2011; Rocha, 2008). Identification of the *cid* promotor region(s) is required to confirm their status as an operon (Shropshire et al., 2019). Additionally, while the hypothetical rescue factor, *cidA*, is delivered along with the modified sperm of infected male mosquitoes, the proposed modification factor itself, *cidB*, has not been found in these sperm (Beckmann and Fallon, 2013; Shropshire et al., 2019). This suggests that the modification factor may sabotage sperm in a specific manner prior to the jettison of *Wolbachia* during sperm maturation (Clark et al., 2002), and the rescue factor restores sperm function but may not directly interact with the modification factor (Shropshire et al., 2019). Alternatively, given that *cidB* shows a six-fold lower expression level than *cidA*, the methods used for protein detection in sperm may not have captured a low-concentration protein like *cidB* (Beckmann and Fallon, 2013; Lindsey et al., 2018).

While their status as a true toxin-antidote system remains up for debate, the *w*Pip *cid* genes and their homologues in the *w*Mel *Wolbachia* strain infecting *D. melanogaster* were confirmed in separate studies to induce CI-like mortality when co-expressed in the testes of uninfected flies (Beckmann et al., 2017; LePage et al., 2017). The CI-like mortality caused by *cidA-cidB* expression was also rescued in crosses between transgenic males and *Wolbachia*-infected females (LePage et al., 2017). Lastly, the hypothesized role of *cidA* as the rescue factor was functionally confirmed via transgenic rescue crosses between *Wolbachia*-infected males and uninfected females expressing *cidA* (Shropshire et al., 2018). Throughout the rest of this section, we will refer to these the CI genes as "*cif*" (CI factor) genes when discussing general features shared across the different variants, and their specific names, *cid* (harbouring a deubiquitinase domain) or *cin* (harbouring PD-(D/E)xK nuclease domains) when discussing specific variants. Despite major advances in the genetics of CI, the mechanism(s) of modification and rescue is/are still unknown. For example, *cidA*-bound *cidB* retains the deubiquitinase activity required for CI-induced mortality, suggesting that, despite the high binding affinity for homologous *cif* protein pairs, this binding is not directly responsible for CI rescue (Beckmann et al., 2017). One model of rescue proposes that, rather than directly inhibiting the activity of the *cifB* toxin, the rescue factor, *cifA*, changes its cellular localization, or blocks its ability to interact with host proteins (Beckmann et al., 2017; Chen et al., 2019).

While studies using heterologous expression of *Wolbachia* genes confirmed the identity of CI factors, these studies also raised new mechanistic questions, including why the recapitulation of the modification step of CI in uninfected hosts requires co-expression of both *cifB* and *cifA*, when *cifB* is the hypothetical modification factor. The requirement for both *cifB* and *cifA* suggests an added level of complexity to the hypothesized CI mechanism, whereby both genes may be involved in modification, while only *cifA* is necessary for rescue (Shropshire et al., 2018). Alternatively, this apparent requirement for both factors may be an artefact of heterologous expression of the *cifB* toxin. These expression systems can result in much higher expression levels compared to native bacterial expression (Harumoto and Lemaitre, 2018). Heterologous *cifB* expression in the absence of *cifA* could provide abnormally high amounts of the modification factor in sperm that results in inviable sperm (Beckmann et al., 2019). Those sperm capable of fertilization may not have been exposed to the *cifB* factor, potentially masking this gene's role as the sole modification factor. Studies investigating

sperm maturation and viability in *cifB*-expressing *D. melanogaster* could clarify the role of *cifA* and *cifB* in CI modification (Beckmann et al., 2019).

Genome comparisons of various CI-inducing *Wolbachia* have revealed tremendous diversity and copy number of *cif* loci (LePage et al., 2017; Lindsey et al., 2018). Generally, the identity and number of *cifA* and *cifB* copies present in a CI-*Wolbachia* genome relates to the crossing dominance of certain strains, with dominant CI-inducing strains harbouring more copies of the CI genes (Bonneau et al., 2018; LePage et al., 2017; Lindsey et al., 2018). This variability extends to the protein encoding domains within these *cif* genes, which can contain numerous domains of different functions. For example, while the original *cidB* gene identified by Beckmann et al. (2017, 2013) encodes a deubiquitinylating protease essential for CI crossing mortality, other *cifB* homologues encode modules that lack any identifiable protease and instead harbour other sets of protein domains including nucleases, restriction enzymes, and/or DNA repair proteins (Lindsey et al., 2018). Recently, functional tests of PD-(D/E)xK nuclease encoding *cif* variants from the *Culex pipiens*-infecting *w*Pip strain (which also harbours the *cid* variants; Beckmann et al., 2017) demonstrated that the nuclease variants are also capable of causing CI (Chen et al., 2019). Consistent with previous studies testing CI induced by the deubiquitinase-encoding *cid* pair (Beckmann et al., 2017; LePage et al., 2017), the so-called *cinA* and *cinB*, which contains two PD-(D/E)xK nucleases domains, induce CI-like mortality when co-expressed in the germline cells of uninfected male *D. melanogaster* (Chen et al., 2019). Additionally, expression of *cinA* alone in the germline of uninfected female flies mated with *Wolbachia*-infected males results in rescue of natural CI, similar to the *cid* system, in which *cidA* alone rescues CI (Chen et al., 2019; Shropshire et al., 2018). In further agreement with the *cid* system, *cinA* and *cinB* show high binding affinity for each other, but this binding does not inhibit the nuclease activity of *cinB* responsible for the mortality effect, suggesting that the antitoxin operates via some mechanism distinct from disruptive binding (Beckmann et al., 2017; Chen et al., 2019).

Despite the variability of homologues of *cifA* and *cifB*, the cytological effects of *Wolbachia*-CI appear consistent across strains, with CI-induced mortality resulting from the delayed condensation of paternal chromatin at the first mitotic division of the zygote, followed by fatal mitotic defects and chromatin bridging (Fig. 1; Tram et al., 2003; Beckmann et al., 2017;Chen et al., 2019; LePage et al., 2017). Specifically, these defects appear to arise from a delay in the recruitment of the histone complex to DNA of the male pronucleus prior to the onset of the first embryonic mitosis

(Landmann et al., 2009). Given that two variants of the *cif* genes cause similar cytological defects despite encoding different protein domains, it seems that there may be multiple ways in which CI-*Wolbachia* can modify sperm chromatin (Chen et al., 2019; LePage et al., 2017). Further research on the downstream mechanics of these deubiquitinase- and nuclease-based systems will reveal at what point these mechanisms converge to cause similar defects in male chromatin.

Wolbachia is not the only symbiont capable of causing CI. To date, three other intracellular bacteria have been implicated in causing CI in arthropod hosts, although two of these three have been discovered only recently (Hunter et al., 2003; König et al., 2019; Takano et al., 2017). The best-studied of these, *Cardinium hertigii* (Phylum Bacteroidetes), has been found to cause CI in a number of arthropod hosts (Gebiola et al., 2016; Hunter et al., 2003; Nakamura et al., 2012; Nguyen et al., 2017; Wu and Hoy, 2012). Genomic comparisons between CI-*Cardinium* and CI-*Wolbachia* revealed very little gene homology (Mann et al., 2017; Penz et al., 2012), and *Cardinium* lacks the *cif* genes responsible for *Wolbachia*-CI (Lindsey et al., 2018). Given the lack of homology between these two bacteria, it's likely that CI arose independently in these two lineages and operates via a different molecular mechanism (Penz et al., 2012). However, a cytological study of *Cardinium*-CI mortality revealed mitotic defects strikingly similar to those seen in *Wolbachia*-CI (Fig. 1; Gebiola et al., 2017). This is perhaps unsurprising, given the conservation of CI-induced cytological defects across *Wolbachia* strains harbouring different *cif* genes (Tram et al., 2003; Beckmann et al., 2017; LePage et al., 2017; Lindsey et al., 2018). That CI generated by two different lineages of bacteria via different upstream mechanisms results in similar cytological defects also suggests that there are multiple avenues for intracellular symbionts to sabotage the host cell cycle in a CI-like manner.

3. Male-killing

Some strains of manipulative symbionts induce sex-specific offspring mortality, only killing infected male hosts either early in embryonic development or later as juveniles. The elimination of male offspring may reduce resource competition for infected female hosts and, in cases involving cannibalistic hosts, even provide developing females with additional and easily accessible nutrition via their undeveloped brothers (Hurst and Majerus, 1993). Like CI, male-killing is a common manipulative phenotype, with

at least four distinct symbiont lineages containing male-killers (Gherna et al., 1991; Hurst et al., 1992; Hurst et al., 1999a: Hurst et al., 1999b). Male-killers are especially prevalent and well-studied in several lepidopteran (Fukui et al., 2015; Hornett et al., 2006) and coleopteran hosts (Hurst et al., 1992, 1996, 1999a, 1999b; Lawson et al., 2001; Schulenburg et al., 2001; Werren et al., 1994) species, although male-killing strains of *Wolbachia* and another prevalent manipulative symbiont, *Spiroplasma*, occur naturally in several *Drosophila* fly species as well (Haselkorn, 2010). The natural occurrence of these two male-killing symbionts in the *Drosophila* model system, as well as the presence of a third male-killer, *Arsenophonus*, in *Nasonia vitripennis*, a parasitic wasp model system, has allowed for more intensive research into the genetic mechanisms controlling symbiont-induced male mortality.

The male-killing phenotype of *Drosophila*-infecting *Spiroplasma* is perhaps the best understood mechanistically of any reproductive manipulation. In the context of the XX/XY sex chromosome system of *Drosophila*, male-specific mortality arises from widespread apoptosis caused by improper X chromosome segregation (Bentley et al., 2007; Harumoto et al., 2016), genome-wide gene mis-expression (Cheng et al., 2016), and widespread neural defects in the central nervous system of male embryos (Martin et al., 2013; Tsuchiyama-Omura et al., 1988). The male-killing phenotype involves the manipulation of the dosage compensation machinery required for proper X chromosome gene expression in XY males, particularly a protein-RNA complex called male-specific lethal (MSL; Veneti et al., 2005; Harumoto et al., 2016). Normally, in the process of dosage compensation, the MSL complex is specifically recruited to the X chromosome in males, where it plays a role in overexpression of X chromosome genes (Penalva and Sánchez, 2003). *Spiroplasma*-infected males show improper localization of the MSL complex; rather than associating only with the single X chromosome of male hosts, the MSL complex also associates with autosomal chromosomes, resulting in altered levels of histone acetylation and overexpression of autosomal genes (Cheng et al., 2016). That *Spiroplasma* causes female mortality in transgenic *Drosophila* that express MSL genes and does not cause mortality in males with non-functional MSL, offers additional strong support for the MSL as the host target for the male-killing mechanism (Cheng et al., 2016; Harumoto et al., 2016; Veneti et al., 2005).

Most recently, a gene for *Spiroplasma* male-killing was identified and functionally confirmed to induce male-specific mortality via expression in uninfected *D. melanogaster* (Harumoto and Lemaitre, 2018). The expression of this plasmid encoded gene, *Spaid* (*S. poulsonii* androcidin), resulted in

male-specific embryonic mortality, phenotypically consistent with male mortality due to *Spiroplasma* infection (Harumoto and Lemaitre, 2018). The *Spaid* gene contains an ankyrin and deubiquitinase domain, and expression of *Spaid*-constructs lacking either domain showed altered male-killing phenotypes with reduced efficacy (Harumoto and Lemaitre, 2018). Specifically, expression of the construct lacking the ankyrin domain did not result in male mortality and the construct did not localize with the MSL complex as observed for wild-type *Spaid*. Constructs without the deubiquitinase domain also showed reduced male mortality, and a lack of association between these constructs and host nuclei. Harumoto and Lemaitre (2018) conclude that the ankyrin domain of *Spaid* is required for its interaction with the host MSL complex, while the deubiquitinase domain is involved in the initial nuclear localization of *Spaid*.

The genome of the *D. melanogaster*-associated strain of male-killing *Spiroplasma* contains a single gene with a recognizable ankyrin domain: *Spaid* (Harumoto and Lemaitre, 2018). In contrast, two other widespread manipulators, *Wolbachia* and *Cardinium,* contain numerous genes with ankyrin domains, which are typically involved in interactions with eukaryotic proteins. Due to their role in eukaryotic protein interactions, ankyrin domain genes were the first genes suspected to be involved in reproductive manipulation (Fenn and Blaxter, 2006; Penz et al., 2012; Sinkins et al., 2005; Yamada et al., 2011). Interestingly, *Wolbachia* also contains a gene similar to *Spaid* containing an ankyrin and deubiquitinase domain. However, expression of this gene does not induce male-killing in uninfected flies (Yamada et al., 2011). More recently, a phage-associated gene has been linked to the male-killing phenotype of *Wolbachia* (Perlmutter et al., 2019). Expression of this gene, *wmk* (*WO-mediated killing*), induces a male-killing phenotype in uninfected *D. melanogaster* consistent with the phenotypes observed in natural male-killing *Wolbachia–Drosophila* symbioses (Perlmutter et al., 2019).

The candidate factors responsible for male-killing in *Wolbachia* and *Spiroplasma*, *wmk* and *Spaid*, respectively, while both found on extrachromosomal elements (a phage and a plasmid) are not homologous (Harumoto and Lemaitre, 2018; Perlmutter et al., 2019). While *Spaid* encodes an ankyrin and deubiquitase domain, *wmk* is predicted to encode two helix-turn-helix domains and an XRE DNA-binding domain (Harumoto and Lemaitre, 2018; Perlmutter et al., 2019). Given the lack of homology of these two factors, *Wolbachia* and *Spiroplasma* likely utilize different mechanisms to induce male-killing. However, these mechanisms seem to converge at their eukaryotic target: the dosage compensation system required for proper

X chromosome gene expression in male hosts (Fukui et al., 2015; Harumoto et al., 2018; Perlmutter et al., 2019). In a 2015 study of male-killing *Wolbachia* in the Lepidopteran host, *Ostrinia furnaculis*, Fukui et al. (2015) found that *Wolbachia* reduced expression of *masculinizer* (*masc*), a dosage compensation factor required for successful male development. Notably, Lepidoptera harbour a ZZ/ZW sex determination system, in which males are the homozygous sex (Kiuchi et al., 2014). In healthy ZZ males, the *masc* protein is involved in the repression of *Z*-encoded gene expression, acting as a reverse type of dosage compensation compared to XX/XY species (Kiuchi et al., 2014). Experimental reduction of *masc* expression in *Bombyx mori* results in male-specific mortality with a similar phenotype as *Wolbachia*-induced mortality of *O. furnaculis* (Fukui et al., 2015). In the *masc*-depleted, doomed males of either species, mortality is associated with overexpression of Z chromosome genes, consistent with the dosage compensation role of *masc* (Fukui et al., 2015). Furthermore, *Wolbachia*-induced male *O. furnaculis* mortality is rescued when male embryos are injected with additional *masc* transcripts, offering further support for *masc* being the target for *Wolbachia* male-killing in *O. furnaculis* (Fukui et al., 2015). Perlmutter et al. (2019) found evidence that *Wolbachia*'s male-killing factor, *wmk*, also targets the dosage compensation system in *Drosophila*. Given the relative conservation of *wmk* across male-killing *Wolbachia* strains, it's quite possible that the *wmk* gene is also responsible for *masc* suppression in *O. furnaculis* (Fukui et al., 2015; Perlmutter et al., 2019).

While *Spiroplasma* and *Wolbachia* use different genes to initiate the male-killing phenotype, their shared target in the dosage compensation system of males seems to result in similar cytological defects responsible for embryonic mortality. Both result in segregation defects during cell division and widespread embryonic apoptosis, although *Wolbachia*-killed males do not exhibit the neural defects found in *Spiroplasma*-killed males (Harumoto et al., 2018).

A third male-killing bacterium, *Arsenophonus*, utilizes a unique strategy to kill male hosts of the parasitic wasp, *Nasonia vitripennis* (Ferree et al., 2008). In this haplodiploid system, *Arsenophonus* selectively kills haploid male embryos by targeting and preventing the formation of the maternally-derived centrosomes that are required for male development in haplodiploid species (Fig. 1; Tram and Sullivan, 2000; Ferree et al., 2008). Loss of these centrosomes results in disorganization of spindle required for functional mitosis and leads to asynchronous mitosis and chromosome condensation, resulting in male-specific embryonic mortality (Ferree et al., 2008). This maternally-derived centrosome inhibition is also observed in *Arsenophonus*-infected female hosts, however female haplodiploid organisms

preferentially use the paternal set of centrosomes for mitosis and so are unharmed (Ferree et al., 2008; Tram and Sullivan, 2000). In an elegant experiment, Ferree et al. (2008) tested the centrosome hypothesis by using a second selfish genetic element, the selfish host chromosome, Paternal Sex Ratio (PSR). PSR causes a male-skewed sex ratio by destroying the paternal genome in diploid eggs, resulting in haploid, incipient male, embryos. PSR also bypasses the requirement for maternal centrosomes in male development; instead, males inherit the paternal centrosomes which remain after the paternal genome is destroyed (Nur et al., 1988). Crosses between *Arsenophonus*-infected females and PSR-bearing males resulted in normal broods of males and females, providing evidence that this symbiont specifically targets maternal centrosomes to kill males (Ferree et al., 2008). The genes responsible for this phenotype are not yet characterized, although assembly of the *Arsenophonus nasoniae* genome has revealed an abundance of suspected toxins, a Type III secretion system, and a potentially functional polyketide synthase system which produces small peptides capable of transmembrane travel, any of which may play a role in its son-killing phenotype (Wilkes et al., 2010).

Finally, male-killing symbionts may target sex determination systems, and in this way be related to feminizers, or even parthenogenesis-inducers. For example, a *Wolbachia* strain, initially described as a feminizer, was found to cause female-skewed sex ratios via late-stage larval male mortality in the lepidopteran host, *Ostrinia scapulalis* (Kageyama et al., 2002; Kageyama and Traut, 2004). This mortality seems to arise from male expression of female isoforms of *doublesex* (*dsx*), a major gene controlling the sex determination system of many insects (Sugimoto and Ishikawa, 2012). Furthermore, this *Wolbachia* is now required for female *dsx* expression, and female hosts cured of *Wolbachia* die as larvae (Sugimoto and Ishikawa, 2012). Given that the sex determination and dosage compensation systems are distinct host targets, this *Wolbachia* strain in *O. scapulalis* seems to be capable of yet another means of male-killing.

While the mechanisms by which *Spiroplasma*, *Wolbachia*, and *Arsenophonus* induce male-specific mortality in their insect hosts have received the bulk of research attention, there are more male-killing bacterial lineages for which the male-killing mechanism is almost entirely unknown. Notable among these lesser studied male-killers are *Rickettsia* and *Flavobacterium*, both of which have been found in various beetle hosts, including many ladybird species (Hurst et al., 1992; Werren et al., 1994; Hurst et al., 1996; Hurst et al., 1999a, 1999b; Lawson et al., 2001; Schulenburg et al., 2001). These symbionts may

target the dosage compensation system, like *Wolbachia* or *Spiroplasma*, or utilize a different strategy like the parasitoid-infecting *Arsenophonus*. Further research on these less understood symbioses and mechanisms will likely reveal a diverse array of strategies utilized by manipulative symbionts to kill male hosts.

4. Feminization

Symbiont-induced reproductive sabotage does not always result in male mortality. Some manipulative symbionts can modify the host phenotype to produce genetic males that are phenotypically female, and thus fully capable of spreading the symbiont (Werren et al., 2008). These feminizing microbes are not restricted to insects and chelicerates, and are also described in crustaceans (Bouchon et al., 1998; Dunn et al., 1993). Nor are these manipulative saboteurs strictly bacteria; several instances of feminizing microsporidia and other parasitic eukaryotes have been described in shrimp species like *Gammarus duebeni* (Dunn et al., 1993; Ironside et al., 2003; Terry et al., 1998; Pickup and Ironside, 2018). Crustaceans and insects have evolved distinct mechanisms for phenotypic sex determination, suggesting that, like those causing CI and male-killing, feminization symbionts can potentially interact with multiple distinct host sex determination pathways (Legrand et al., 1987; Martin et al., 1999; Verhulst et al., 2010; Verhulst and van de Zande, 2015).

In many crustaceans, male development is controlled by a specialized androgenic gland (Cordaux and Gilbert, 2017). This male-associated gland appears to be a common target for multiple crustacean-associated feminizing microbes, including the microsporidian, *Nosema,* which inhibits androgenic gland development (Rodgers-Gray et al., 2004). Another feminizing crustacean symbiont, *Wolbachia*, targets the androgenic gland in a similar manner, inhibiting its development and resulting in expression of the female phenotype in genetic male hosts of the pillbug, *Armadillidium vulgare* (reviewed in Cordaux and Gilbert, 2017). *Armadillidium vulgare* has a ZZ/ZW sex chromosome system, so that feminized males (ZZ) produce more feminized males via *Wolbachia* transmission (Cordaux and Gilbert, 2017). Over time, this has led to the loss of the W chromosome responsible for female development and complete dependence on *Wolbachia* for female production and population persistence (Cordaux and Gilbert, 2017).

Interestingly, studies investigating *A. vulgare* feminization found that certain ZZ individuals that were not infected with *Wolbachia* also exhibited a feminized phenotype ("f" females; Cordaux and Gilbert, 2017). These feminized but *Wolbachia*-free females were found to harbour a large nuclear insert (~3 Mb) thought to be responsible for the phenotypic switch to

female development (Cordaux and Gilbert, 2017; Leclercq et al., 2016). In a striking example of horizontal gene transfer between a symbiont and its host, genomic analysis of the "f element" found homology with the feminizing *Wolbachia* strain found in *A. vulgare*, *w*VulC (Leclercq et al., 2016).

While it is still unclear whether a gene (or genes) within this insert is responsible for female development, or whether the insertion disrupted a gene required for male development, the gene contents of the f element provided an initial list of 3000 potential feminizing gene candidates (Leclercq et al., 2016). By comparing the genome of the feminizing *Wolbachia* strain *w*VulC, the source of the f element, with a closely related CI *Wolbachia* strain, Badawi et al. (2018) have narrowed this list of candidates to 35 genes. Expression profiles of these candidates throughout isopod development revealed two genes of particular interest: a gene lacking any known domain, *w*Vul_1821, and one containing an ankyrin and a protease domain, *w*Vul_1408 (Badawi et al., 2018). Curiously, *w*Vul_1408 is absent in the f element, suggesting this gene is not involved in feminization in f element-bearing individuals (Badawi et al., 2018).

Much of the work on feminizing symbionts has focused on crustacean-infecting microbes, culminating in a short list of feminizing candidate genes. However, the sex determination systems of crustaceans and insects are fundamentally different (Legrand et al., 1987; Verhulst and van de Zande, 2015). Crustacean sex determination is hormonally based and involves the androgenic gland; insect sex determination occurs on a cell by cell basis, involving a cascade dependent on sex-specific splicing variants of one or more transcripts (Verhulst and van de Zande, 2015). Notable among these genes are *doublesex*, the final gene in the cascade responsible for regulating sex-specific gene expression, and immediately above it, *transformer* (*tra*), a paralog of *dsx*, which controls *dsx* expression (Verhulst et al., 2010; Verhulst and van de Zande., 2015). Further upstream genes vary depending on insect species; however, the basal elements of the sex determination cascade, *dsx* and *tra*, are found throughout insects (Verhulst et al., 2010; Verhulst and van de Zande, 2015; Geuverink and Beukeboom, 2014). Given the massive differences between sex determination of these two major arthropods groups, it is likely that feminization arises from different mechanisms in crustacean- and insect-infecting feminizers.

In insects, *Wolbachia* is the most common feminizing symbiont, with feminizing strains identified in both lepidopteran hosts and a planthopper species (Hiroki et al., 2002; Narita et al., 2007; Narita et al., 2011; Negri et al., 2006). Notably, a feminizing *Wolbachia* found in the butterfly host, *Eurema mandarina*, is required for female development (Kageyama et al., 2017). Infected

E. mandarina have a ZZ/ZO sex chromosome system, rather than the typical ZZ/ZW system of lepidopterans; this host lacks the W chromosome responsible for female sex determination in many butterflies and moths. Like the *Wolbachia* in *A. vulgare*, the feminizing *Wolbachia* strain in *E. mandarina* seems to have replaced the nuclear sex determination system of its host (Kageyama et al., 2017). Antibiotic treatment of *E. mandarina* larvae induces expression of the male *dsx* isoform and results in intersex individuals. A similar result is seen when *Wolbachia*-infected larvae of *E. hecabe* (sister species to *E. mandarina*) are fed antibiotics (Narita et al., 2007). These results suggest that feminizing *Wolbachia* may target the sex determination cascade in insect hosts, given that the feminizing influence of *Wolbachia* is required throughout host development and similarly, insect sex determination occurs constantly on a cell-by-cell basis throughout insect development (Narita et al., 2007; Kageyama et al., 2017; Verhulst and van de Zande, 2015). In this set of experiments, antibiotic treatments also resulted in high rates of late-stage (pupal) host mortality, consistent with defects caused by disruption of the sex determination system (Kageyama et al., 2017; Narita et al., 2007).

A different strain of *Wolbachia*, now considered to be a late-stage male-killer, is also required for female development of the moth, *Ostrinia scapulalis* (Kageyama et al., 2002; Kageyama and Traut, 2004). While eventual loss of the W chromosome via the production of ZZ female offspring of feminized males is expected, loss of the female W chromosome leading to an obligatory requirement for a symbiont in a male-killing system is a surprising result. Intriguingly, this male-killer of *O. scapulalis* utilizes a seemly different technique from other *Wolbachia* to kill males by manipulating the sex determination cascade, resulting in a mismatch between host genotype and the splicing variant *dsx* it expresses (Sugimoto and Ishikawa, 2012). It is possible that the male-killing *Wolbachia* in *O. scapulalis* is a feminizing strain "gone bad." This could explain the manipulation of the sex determination system that results in male mortality, and the unlikely requirement of *Wolbachia* presence for female development. Genomic comparisons between the *O. scapulalis* strain and both feminizing and male-killing *Wolbachia* strains of other hosts may provide some insight into this strange male-killer, as well as the into the genetic basis of feminization in insect hosts.

Aside from *Wolbachia*, only one other symbiont has been documented to cause feminization of an arthropod host. One strain of *Cardinium* was shown to feminize a species of the haplodiploid *Brevipalpus* mite (Groot and Breeuwer, 2006; Weeks et al., 2001). *Cardinium* infection results in entirely haploid female mites, indicating that this *Cardinium* feminizes haploid males,

and as the authors point out, is perhaps one of the few if not only examples of an entirely haploid metazoan (Groot and Breeuwer, 2006). Unfortunately, little else is known concerning the mechanism of this second bacterial symbiont's feminization. Homologues of *dsx*- and *tra*- have been identified in another mite species, *Metaseiulus occidentalis*, suggesting that the sex determination system of mites, and a potential host target for feminizing symbionts, may be similar for chelicerates and insects (Pomerantz et al., 2015).

5. Parthenogenesis induction

A fourth reproductive manipulation by heritable bacteria is the induction of parthenogenesis, in which the infected female host asexually produces genetically female offspring. Parthenogenesis-inducing (PI) symbionts have been described in numerous haplodiploid arthropod lineages, including parasitic wasps (Giorgini et al., 2010; Gottlieb et al., 2002; Pannebakker et al., 2004; Stouthamer et al., 1993; Zchori-Fein et al., 1995; Zchori-Fein et al., 2001), thrips (Arakaki et al., 2001), and some mite species (Weeks and Breeuwer, 2001). There are also instances of PI-associated bacteria occurring in diplodiploid hosts such as Collembola, booklice, armoured scales, and many beetle species (Pike and Kingcombe, 2009; reviewed in Ma and Schwander, 2017). Unfortunately, experimental confirmation of symbiont-induced parthenogenesis is difficult in these diplodiploid systems, as the symbiont is often required for host reproduction and is not amenable to typical manipulation strategies using antibiotics or temperature (Graber and Fallon, 2019; Ma and Schwander, 2017). The symbionts in these instances often remain phenotypically uncharacterized, and most of our present mechanistic understanding of PI results from studies of PI symbionts in haplodiploid hosts, typically parasitic wasp species.

To date, three symbiont lineages have been confirmed to cause PI in arthropod hosts (Ma and Schwander, 2017). The most widespread of these, *Wolbachia*, causes PI in numerous arthropod species, including minute parasitoid wasps in the genus *Trichogramma*, in which symbiont-induced PI was first confirmed (Stouthamer et al., 1990; Stouthamer and Werren, 1993). Parthenogenesis induction has also been shown to be caused by *Cardinium* in the parasitic wasp genus *Encarsia* (Zchori-Fein et al., 2004) and is associated with parthenogenesis in some armoured scale species (Gruwell et al., 2009; Provencher et al., 2005). Lastly, two lineages of

Rickettsia have been shown to cause PI in eulophid (Chalcidoidea) parasitoid species (Giorgini et al., 2010; Hagimori et al., 2006).

The mechanism of PI has been particularly challenging to study and is consequently less well understood compared to other reproductive manipulations. One challenge is that hosts infected with a PI symbiont have a tendency to become reliant on these symbionts for reproduction, such that sexual reproduction cannot be restored, which would allow for rigorous comparisons between infected and uninfected individuals. Attempts to cure the host of the symbionts often leads to reproductive failure either through production of infertile male offspring, sexual fertilization failure, and/or egg development failure (Giorgini et al., 2007; Graber and Fallon, 2019; Russell and Stouthamer, 2011; Timmermans and Ellers, 2009; Zchori-Fein et al., 1995). Secondly, PI symbionts are not known from a model insect such as *Drosophila,* or mosquitoes, or even the model hymenopteran parasitoid *Nasonia vitripennis*. Instead, many PI symbionts occur in especially minute species of endoparasitoid wasps (e.g., *Trichogramma* and *Encarsia)*, further restricting the ability of researchers to manipulate these systems (Adachi-Hagimori et al., 2008; Giorgini et al., 2007; Gottlieb et al., 2002; Ma et al., 2015; Pannebakker et al., 2004; Stouthamer and Kazmer, 1994; Zchori-Fein et al., 1995). Finally, PI symbionts manipulate reproduction primarily in the hymenopteran superfamily Chalcidoidea, in which the normal mechanism of sex determination is not well understood (Van Wilgenburg et al., 2006). Thus, researchers have a doubly difficult challenge of understanding what pieces of the (unknown) sex determination system of chalcidoids are targeted by PI symbionts.

In spite of these difficulties, cytological analysis of egg and early embryonic development and microsatellite marker analysis (Ma and Schwander, 2017) have provided some insights into mechanism. These studies of symbiont-infected parthenogenetic eggs have revealed that PI symbionts cause parthenogenetic development via the restoration of haploid eggs to diploidy. While diploidy is unlikely to be sufficient for female development (in chalcidoid species), it is likely to be necessary in most instances (Giorgini et al., 2009; Ma and Schwander, 2017). There appear to be two main mechanistic strategies to restore diploidy: (1) disruption of early mitosis in newly oviposited embryos that causes mitotic products to coalesce again, known as gamete duplication (Gottlieb et al., 2002; Pannebakker et al., 2004; Stouthamer and Kazmer, 1994), and (2) disruption of the reductive division of meiosis, resulting in a diploid zygote prior to the beginning of mitosis (Adachi-Hagimori et al., 2008; Giorgini et al., 2007). These two types of

diploidy restoration can have different effects on host heterozygosity, depending on when they occur within oogenesis and whether they allow for recombination (reviewed in Ma and Schwander, 2017).

The first cytological study of *Wolbachia*-PI, in *Trichogramma* wasps, revealed that diploidy restoration was the result of gamete duplication (Stouthamer and Kazmer, 1994). In this system, the egg cells undergo typical meiosis following oviposition, with three of the four chromatid products condensing into polar bodies, and the fourth becoming a haploid maternal pronucleus (chromosome $n = 5$). Upon oviposition, this unfertilized, haploid egg, which would develop as a male in a sexual species, prepares for the first mitotic division. However, during mitotic anaphase, no spindle forms, the chromatid pairs segregate only slightly, and the nucleus does not divide, resulting in a diploid daughter cell ($2n = 10$ chromosomes) (Fig. 1; Stouthamer and Kazmer, 1994). This type of diploidy restoration results in offspring that are homozygous at every locus. This finding appears to explain why PI *Wolbachia* has rarely been found in the superfamily of wasps Ichneumonoidea. In this superfamily, the dominant sex determination mechanism is complementary sex determination (CSD), whereby heterozygosity at any one of a number of sex determination alleles triggers female production, while hemizygosity results in males, as does high levels of homozygosity in diploid individuals (resulting in diploid, generally sterile males) (Zhishan et al., 2003). CSD does not appear to occur in all ichneumonoids, however, including the braconid *Asobara tabida* (Beukeboom et al., 2000), which also harbours a PI-inducing strain of *Wolbachia* (Ma et al., 2015). The absence of CSD may explain the presence of PI *Wolbachia* in this host (Ma et al., 2015).

Gamete duplication has been described in other instances of *Wolbachia*-PI via either cytology (Giorgini et al., 2007; Gottlieb et al., 2002; Pannebakker et al., 2004) or has been inferred by measuring heterozygosity via microsatellite markers (Plantard et al., 1998). However, there is variation in the timing of the aberrant mitosis step within gamete duplication, as seen in the PI-*Wolbachia*-infected parasitoid, *Muscidifurax uniraptor*. In this host, the initial mitosis cycle occurs normally, and instead the two resulting nuclei from the initial mitotic event fuse together during the early stages of the subsequent mitosis cycle, resulting in diploidy restoration (Gottlieb et al., 2002). This contrasts with the typical gamete duplication at the first mitotic division of *Trichogramma* wasps (Stouthamer and Kazmer, 1994) as well as in other parasitoid hosts, *Leptopilina clavipes* (Pannebakker et al., 2004) and *Encarsia formosa* (Giorgini et al., 2007). A similar process of nuclear fusion after the

first mitotic division was observed in the parthenogenetic gall wasp, *Diplolepis rosae* (Stille and Dävring, 1980). However, while *D. rosae* does harbour *Wolbachia*, it has not been confirmed as the causative agent for PI in this host (Van Meer et al., 1999). Unfortunately, given that there are only a few detailed cytogenetic studies of *Wolbachia*-PI, is it not clear at this point whether these variations of gamete duplication are a result of genetic differences in PI symbiont strain, titre effects, or are due to expression of the PI phenotype in different host environments.

The two lineages of *Rickettsia* that cause PI occur in chalcidoid wasp species in the family Eulophidae, including *Neochrysocharis formosa* and *Pnigalio soemius* (Giorgini et al., 2010; Hagimori et al., 2006). Cytogenetic studies of *Rickettsia*-infected *N. formosa* found that this symbiont restores diploidy prior to mitosis, such that *N. formosa* eggs are functionally diploid because the reduction division of meiosis does not occur. Instead the eggs undergo just one round of divisional meiosis prior to the first mitotic division (Adachi-Hagimori et al., 2008). The final PI symbiont, *Cardinium*, appears widespread in another chalcidoid wasp lineage, the Aphelinidae, and was shown to cause PI in *Encarsia hispida* (Giorgini et al., 2007; Giorgini et al., 2009; Zchori-Fein et al., 2001; Zchori-Fein et al., 2004). Cytogenetic analysis of *Encarsia* eggs revealed a third, unique, mechanism of diploidy restoration that acts pre-mitotically (Giorgini et al., 2007). In this system, the first meiotic division is functionally aborted entirely, resulting in the egg cells undergoing only a single round of meiotic division resulting in diploid, rather than haploid eggs (Giorgini et al., 2007). Similar to the temporal variation observed in *Wolbachia*-induced gamete duplication, certain *Cardinium* strains seem to induce the aborting of meiotic division at different times. In *E. hispida* and *E. guadeloupe*, *Cardinium* causes the first meiotic division to fail, while in *E. tabacivora (= E. pergandiella*), the first meiotic division occurs normally, however the two resulting nuclei fuse after this first round of meiosis (Giorgini et al., 2007). Both strategies, named central fusion, result in diploid egg cells. Given that all three lineages of PI symbionts restore diploidy through different mechanisms, it's likely that this manipulative strategy has convergently evolved multiple times in symbiotic bacteria.

These various cytological studies have at times revealed a fascinating secondary result: the production of diploid male wasps from PI symbiont-infected mothers exposed to antibiotics (Giorgini et al., 2009; Ma et al., 2015). These PI symbionts typically are found in haplodiploid systems, in which normal sexually produced males are haploid and diploid male production is aberrant (Giorgini et al., 2009). Examples of diploid male production

have been observed in symbioses involving both *Wolbachia* and *Cardinium*, although it appears male diploidy does not occur in every instance of these PI symbioses (Giorgini et al., 2009; Ma et al., 2015). For example, while *Cardinium*-infected *E. hispida* females will produce diploid male progeny after exposure to antibiotics, other closely related host species like *E. guadeloupe* and *E. tabacivora* do not produce males at all and are rendered effectively sterile by antibiotics (Giorgini et al., 2007, 2009). It has been hypothesized that the diploid males of *E. hispida* result from relaxed selection on the host's production of normal haploid males via long-term infection with the PI-*Cardinium*, rather than *Cardinium* itself, however this has not yet been tested (Giorgini et al., 2009). However, diploid males are produced when their adult mothers are fed antibiotics. Hence, it is possible that bacterial products, whether live or dead, that remain in the egg are sufficient to induce diploidy, as seen in another parasitoid, *Telenomus nawai* (Arakaki et al., 2000). Alternatively, *Cardinium* could set in motion a developmental programme in the host pupal stage that eventually leads to diploidy of eggs, but performs a second, feminizing step within the oviposited egg. Antibiotic treatment then interrupts the second process, but not the first. Indeed, evidence from the mite-infecting feminizing strain of *Cardinium* shows that this symbiont feminizes haploid individuals independent of diploidy restoration (Groot and Breeuwer, 2006). Experiments designed to reduce *Cardinium* density prior to adult emergence, perhaps by exposing larval or pupal *E. hispida* to stressful temperatures (Doremus et al., 2019) or microinjected antibiotics, may help elucidate whether this symbiont feminizes host-diploid males or causes PI via a two-step diploidy-inducing and feminizing mechanism.

Wolbachia-infected *Asobara japonica* (Braconidae: Ichneumonoidea) females fed antibiotics also produce diploid males, although in this case, diploidy seems to result from a low-density *Wolbachia* infection (Ma et al., 2015). When *A. japonica* eggs retain a high-density *Wolbachia* infection they develop as diploid female parthenogens, while a low-level infection results in diploid males, and the absence of *Wolbachia* results in typical haploid male development (Ma et al., 2015). Another study found that antibiotic treatment of *Wolbachia* in *Trichogramma* leads to diploid male production (Tulgetske, 2010). These studies suggest that, like *Cardinium*, *Wolbachia*-induced PI may also proceed in a two-step fashion, in which *Wolbachia* first restores host diploidy, then performs a second, feminizing step to induce female development (Ma et al., 2015; Tulgetske, 2010). This could mean that PI and feminizing *Wolbachia* and *Cardinium* perform similar feminizing actions via the sex

determination system, but PI symbionts also perform the additional diploidization step (Kageyama et al., 2017; Ma et al., 2015; Sugimoto and Ishikawa, 2012; Weeks et al., 2001). Unfortunately, we do not presently know the bacterial factors responsible for either mechanism, and this connection between feminization and PI remains speculative.

Outside of these cytogenetic studies, little is known concerning the mechanism of symbiont-induced parthenogenesis. Complete genomes are only available for a handful of PI-*Wolbachia* strains (Faddeeva-Vakhrusheva et al., 2017; Lindsey et al., 2016), and no genomes for PI-*Rickettsia* or PI-*Cardinium* have been published. A genomic comparison between a PI strain and other *Wolbachia* genomes did not reveal clear candidates for the bacterial factors responsible for PI (Lindsey et al., 2016). Notably, the PI strain used in this comparative analysis, a *Wolbachia* from *Trichogramma pretiosum*, (wTpre) seems to lack the WO bacteriophage that encodes both the CI and male-killing factors found in other *Wolbachia* strains (Gavotte et al., 2006; Lindsey et al., 2016). While other PI strains of *Wolbachia* do harbour the WO phage, it's absence in the genomes of several PI strains suggests that the phage does not play a direct role in parthenogenesis induction (Gavotte et al., 2006). The *w*Tpre genome also shows a widespread abundance of truncated versions of *Wolbachia* genes (Lindsey et al., 2016). A second *Wolbachia* strain, thought to be responsible for PI in the diploid collembolan host, *Folsomia candida*, harbours the largest recorded *Wolbachia* genome and also harbours many genes encoding various ankyrins (Pike and Kingcombe, 2009; Faddeeva-Vakhrusheva et al., 2017). Given the scarcity of PI genomes available, and the variation in overall genomic decay observed in PI-*Wolbachia* genomes, it is unclear whether the generalized abundance of truncated genes observed in *w*Tpre is unique to this *Wolbachia* strain or related strains from *Trichogramma*, or a more general hallmark of PI strains. The genome, like those of other *Wolbachia*, also contains many genes encoding products with ankyrin domains, which may play a role in host interaction (Lindsey et al., 2016). However, until more PI genomes are available, the bacterial factor(s) responsible for *Wolbachia*-PI is likely to continue to elude researchers.

6. Manipulative symbionts can induce multiple phenotypes

While the symbionts detailed in this review are usually denoted by the phenotypes they exhibit in their hosts (e.g., male-killers), symbiont lineages

may not be restricted to performing a single type of manipulation. *Wolbachia*, the "master manipulator" has strains causing all four phenotypes (Werren et al., 2008). *Cardinium* can induce three distinct phenotypes (Hunter et al., 2003; Weeks et al., 2001; Zchori-Fein et al., 2001), and *Rickettsia* perform both male-killing and parthenogenesis induction (Hagimori et al., 2006; Hurst et al., 1992). However, any one strain of these bacteria is typically associated with a single phenotype. There have been notable exceptions to this rule, with certain strains of *Wolbachia* spontaneously causing novel phenotypes under different conditions. These instances of novel phenotypes suggest that at least some symbionts contain the genetic infrastructure to induce multiple manipulations.

The most frequent examples of phenotype switching come from male-killing *Wolbachia* that spontaneously show CI phenotypes, or vice versa (Hornett et al., 2008; Jaenike, 2007;Sasaki et al., 2002; Sasaki et al., 2005). In one case, this phenotype shift is the result of experimental transinfections of *Wolbachia* from one host species to another (Sasaki et al., 2002; Sasaki et al., 2005). Upon successful transinfection to the novel host, the *Wolbachia* strain, which originally caused CI, induced male-killing in its novel host (Sasaki et al., 2002; Sasaki et al., 2005). A study by Jaenike (2007) found similar results in *Drosophila* through introgression of CI-*Wolbachia*-infected *Drosophila recens* into the genetic background of a different host species, *D. subquinaria*. While the *Wolbachia* strain caused CI in *D. recens*, the phenotype switched to male-killing in *D. subquinaria* (Jaenike, 2007). Another study using *Drosophila* found that the male-killing phenotype of *Wolbachia* was not only reduced after exposure to high temperatures, but surviving males were infected with *Wolbachia* and exhibited CI (Hurst et al., 2000). There are even examples of spontaneous phenotype switching in natural host populations infected with male-killing *Wolbachia* (Hornett et al., 2008). Host resistance to the male-killing phenotype has been shown to arise in populations, likely due to the intense conflict that arises between the nuclear genome and the male-killing symbiont (Hornett et al., 2006; Hurst et al., 1992; Majerus and Majerus, 2010). In theory, the resistance mutation should spread quickly through the host population, rendering the male-killing strategy ineffective (Hurst et al., 1992). This was documented in *Hypolimnas* butterflies in the South Pacific, but interestingly, when the *Hypolimnas* host evolved resistance to male-killing, the *Wolbachia* that was formerly male-killing was then shown to cause CI (Hornett et al., 2008). Interestingly, these transitions seem to show comparable trends. When a CI strain is moved to a novel host, it may now induce a

male-killing phenotype; when male-killing fails, the surviving infected males undergo CI modification.

These studies suggest that *Wolbachia* can contain the genetic tools to induce multiple phenotypes. Indeed, another study investigating *Drosophila pandora* found that females infected with male-killing *Wolbachia* could mate compatibly with males infected with a CI-*Wolbachia*, indicating that expression of one phenotype does not prevent a symbiont from expressing other aspects of different manipulative phenotypes (Richardson et al., 2016). The discovery of the *Wolbachia* genes responsible for both male-killing and CI, and the presence of both gene sets in *Wolbachia* strains, provides direct evidence that a single *Wolbachia* strain may harbour genes responsible for at least these two manipulations (Beckmann et al., 2017; LePage et al., 2017;Lindsey et al., 2018; Perlmutter et al., 2019). The genes responsible for CI, *cifA* and *cifB*, and male-killing, *wmk*, are both associated with the WO phage that is widespread throughout the *Wolbachia* (Lindsey et al., 2018; Perlmutter et al., 2019). For example, the CI-inducing strain from *D. melanogaster*, *w*Mel, also has the *wmk* gene and shares high overall genome similarity with the male-killing strain, *w*Rec from *D. recens*, raising the possibility that CI inducing strains like *w*Mel could potentially cause male-killing in novel host environments (Jaenike, 2007; Perlmutter et al., 2019).

Why certain *Wolbachia* impose a male-killing phenotype upon their host rather than a CI phenotype is not clear, although phenotype expression is not under obvious bacterial transcriptional control, as *Wolbachia* appears to express both gene sets at the same relative levels ($cifA > cifB = wmk$), regardless of the resulting phenotype (Perlmutter et al., 2019). Instead the resulting phenotype may be influenced by bacterial post-transcriptional or post-translational control, or be attributed to host genotype, as hosts tend to undergo rapid evolution of male-killing suppression, which has been repeatedly observed in male-killing systems (Hayashi et al., 2018; Hornett et al., 2006; Majerus and Majerus, 2010). For example, *Wolbachia* could express genes for both phenotypes, but in a male host that is resistant to male-killing, male-killing would fail, but if the *cif* genes are expressed, the surviving male would have CI-modified sperm. Such a scenario has occurred in populations of *Hypolimnas* butterflies (Hornett et al., 2008). This two-pronged modification approach, in which male-killing has priority over CI, would better ensure symbiont maintenance, despite host efforts to suppress these often-harmful manipulations. However, it seems strange that these *Wolbachia* strains retain, and even express, a functional *wmk* gene

in resistant hosts, when other unnecessary genes are often lost in the genomes of heritable symbionts (Moran et al., 2008; Perlmutter et al., 2019). Phenotype switching has only been observed thus far in *Wolbachia*, but this may have more to do with the greater number of studied *Wolbachia*-host systems than an inability of other symbionts to induce multiple phenotypes.

7. The eukaryotic cell cycle is a common target of bacterial manipulators

Generally, symbiont-induced manipulations converge upon a common target: the eukaryotic cell cycle (Fig. 1). The only exception to this appears to be feminization, which may target the insect sex determination system in insect hosts and prevent differentiation of the androgenic gland responsible for male development in crustaceans (Narita et al., 2007; Sugimoto and Ishikawa, 2012; Kageyama et al., 2017; Cordaux and Gilbert, 2017). The other mechanisms seem to cause defects in chromatin that results in either malfunctional or modified cell cycle processes. In male-killing, the symbiont targets the dosage compensation system, a protein complex responsible for modifying male gene expression on sex chromosomes (Fukui et al., 2015; Harumoto et al., 2016; Perlmutter et al., 2019; Veneti et al., 2005). This leads to genome-wide altered gene expression, which results in widespread cellular defects that become apparent during embryonic mitosis, in which chromatin bridging in males results in embryonic death (Bentley et al., 2007; Harumoto et al., 2016; Harumoto et al., 2018). The CI strategy also targets host chromatin, however in CI the symbiont-attributed DNA modifications are not conveyed via a sex-specific protein complex (Gebiola et al., 2017; Tram et al., 2003). The actual targets of symbiont-induced CI are not yet identified, although histones and other proteins involved in chromatin wrapping prior to the first mitotic division may be involved (Landmann et al., 2009). These CI modifications also result in chromatin bridging during early embryonic mitosis (Gebiola et al., 2017; Tram et al., 2003). Finally, PI symbionts seem to modify either meiosis or mitosis, although these modifications do not kill the host; instead, PI symbionts either suppress entire cycles of meiosis or cause secondary merging of nuclear products from the cell cycle (Giorgini et al., 2007; Gottlieb et al., 2002; Pannebakker et al., 2004; Stouthamer and Kazmer, 1994). The PI phenotype also does not cause chromatin bridging like CI or male-killing; however, all three mechanisms require that the altered eukaryotic cell

proceeds through the cycle cell. The cell cycle, one of the most conserved elements across eukaryotic species, has internal checkpoints that respond to various forms of DNA and spindle damage (Elledge, 1996). That three of the four symbiont-induced reproductive manipulations are able to both modify the host DNA and prevent detection of these modifications by these checkpoint mechanisms suggests that there may be conserved elements in these manipulations that bypass cell cycle checkpoints.

8. The importance of a manipulator's extrachromosomal parasites

Like their free-living cousins, many heritable symbionts harbour extrachromosomal elements like bacteriophages and plasmids (Bordenstein and Reznikoff, 2005). In several cases, these elements contain genes required for a symbiont-induced phenotype (Ballinger et al., 2019; Degnan and Moran, 2008; Oliver et al., 2009; Beckmann et al., 2017; LePage et al., 2017; Harumoto and Lemaitre, 2018; Perlmutter et al., 2019). These are not limited to reproductive manipulators, indeed there are notable examples of extrachromosomal elements playing direct roles in defensive symbioses (Ballinger and Perlman, 2019; Oliver et al., 2009). For example, certain *Spiroplasma* strains infecting *Drosophila* confer protection against fly natural enemies like nematodes and parasitoids (Ballinger and Perlman, 2017; Jaenike et al., 2010). The ribosome-inactivating toxins (RIPs) responsible for the invading parasite's mortality are often abundant within a *Spiroplasma* genome, with some RIPs located on *Spiroplasma*-associated plasmids (Ballinger and Perlman, 2017, 2019; Hamilton et al., 2016). In another case, an aphid defensive symbiont, *Hamiltonella defensa*, confers protection against aphid parasitoids (Oliver et al., 2005). However, this protective phenotype requires that *H. defensa* harbour the APSE (*Acyrthosiphon pisum* secondary endosymbiont) phage, which encodes toxins that target the developing wasp larva (Brandt et al., 2017; Oliver et al., 2009). In this system, phage loss results in a substantial loss of protection (Oliver et al., 2009), while restoration of phage infection restores the protective phenotype (Brandt et al., 2017; Lynn-Bell et al., 2019).

Extrachromosomal elements also play a major role in several reproductive manipulations. The same *Spiroplasma*-associated plasmid that harbours copies of the ribosome-inactivating toxin also contains *Spaid*, the factor responsible for *Spiroplasma*-induced male-killing in *Drosophila* (Harumoto and Lemaitre, 2018). *Wolbachia* is frequently infected with

the WO phage that contains a region of genes thought to be involved in eukaryote interactions (Bordenstein and Bordenstein, 2016). Among these genes are *wmk*, the *Wolbachia* male-killing candidate factor, and the *cif* CI factors (Beckmann et al., 2017; LePage et al., 2017; Perlmutter et al., 2019).

Given that the extrachromosomal elements of two unrelated symbionts are responsible for at least some of their reproductive manipulations, research into the uncharacterized extrachromosomal elements of other manipulators is enticing. For example, a CI-inducing strain of *Cardinium*, harbours a plasmid that contains numerous genes potentially involved in insect host interaction (Penz et al., 2012). Interestingly, another strain of *Cardinium*, *c*Sfur, infecting the planthopper *Sogatella furcifera*, does not seem to contain an intact plasmid, but does have certain plasmid genes incorporated into its genome (Zeng et al., 2018). While *Cardinium* is known to cause CI in *S. furcifera* (Nakamura et al., 2012), it is not yet clear whether the strain in this study is a CI-inducing strain; furthermore, a whitefly-infecting *Cardinium* strain also harbours the plasmid but does not induce CI, leaving the nature of this plasmid's role in its host bacterium's biology a mystery (Santos-Garcia et al., 2014).

9. Conclusion

The research community investigating bacterial reproductive manipulators has made major strides in the past few decades, culminating with work identifying the bacterial genes responsible for several symbiont-induced manipulations in arthropod hosts (Beckmann et al., 2017; Harumoto and Lemaitre, 2018; LePage et al., 2017; Perlmutter et al., 2019; Shropshire et al., 2018). These and other studies have revealed a fascinatingly diverse array of bacterial lineages capable of manipulating host reproduction, many of which use entirely unique pathways to cause their respective manipulations. However, these studies have also revealed mechanistic themes consistent across the different phenotypes and symbiont lineages. The host cell cycle especially seems to be a consistent target for heritable symbionts prone to manipulation, which is perhaps not surprising given that this fundamental process is regulated by conserved genes across the hosts of these symbionts. These symbionts may target the cell cycle directly, as in the case of male-killing *Arsenophonus* that targets mitotic organelles (Ferree et al., 2008), or indirectly, like another male-killing symbiont, *Spiroplasma*, which sabotages an unrelated protein complex in a

manner that results in an aberrant cell cycle (Harumoto and Lemaitre, 2018). These symbionts also themselves often harbour intracellular passengers like phages and plasmids that are essential for host manipulation. As research on these manipulative cell tenants progresses into the next decade, work on identifying host targets for manipulation will reveal more common themes and distinctions in these mechanisms. As these symbionts, most notably *Wolbachia*, see increased application towards control of arthropod pests, knowledge of these reproductive manipulations is of paramount importance.

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