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Manipulation of Arthropod Sex Determination by Endosymbionts: Diversity and Molecular Mechanisms

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Arthropods · Endosymbiont · Epigenetics · Hormonal signaling · Molecular mechanism · Sex determination · Sexual differentiation

Abstract
Arthropods exhibit a large variety of sex determination systems both at the chromosomal and molecular level. Male heterogamety, female heterogamety, and haplodiploidy occur frequently, but partially different genes are involved. Endosymbionts, such as *Wolbachia*, *Cardinium, Rickettsia,* and *Spiroplasma,* can manipulate host reproduction and sex determination. Four major reproductive manipulation types are distinguished: cytoplasmic incompatibility, thelytokous parthenogenesis, male killing, and feminization. In this review, the effects of these manipulation types and how they interfere with arthropod sex determination in terms of host developmental timing, alteration of sex determination, and modification of sexual differentiation pathways are summarized. Transitions between different manipulation types occur frequently which suggests that they are based on similar molecular processes. It is also discussed how mechanisms of reproductive manipulation and host sex determination can be informative on each other, with a special focus on haplodiploidy. Future directions on how the study of endosymbiotic manipulation of host reproduction can be key to further studies of arthropod sex determination are shown.

Arthropods cover over 1.2 million described species that account for about 80% of all known living animal species. They have colonized virtually all habitats on Earth. In line with this broad adaptation to many conditions, they exhibit an enormous variety of life histories and reproductive modes. They also show surprisingly large variation and turnover in sex determination systems. It is therefore a prime group of organisms to study how changes in sex determination mechanisms come about, a current topic in evolutionary biology that is not well understood. A particular aspect of arthropod biology is their frequent infection with microorganisms that can be mutualistic, parasitic, or commensal. A specific group are endosymbionts, such as *Wolbachia*, *Cardinium, Rickettsia*, *Spiroplasma,* and *Arsenophonus* bacteria, microsporidia and viruses, that manipulate their host's reproduction in a variety of ways [reviewed in Hurst et al., 1996; Werren et al., 2008; Kageyama et al., 2012]. These intracellular parasites are maternally transmitted through

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the egg cytoplasm. As males are an evolutionary dead end for them, any symbiont having the capability to increase female production is at an advantage and can invade host populations [Partridge and Hurst, 1998; Duron et al., 2008; Werren et al., 2008; Cordaux et al., 2011]. This can be realized through causing thelytokous parthenogenesis, male killing, or feminization. As they enhance their own transmission at the expense of their host's fitness, their presence generates genetic conflicts between the 2 sexes and possibly an ensuing co-evolutionary arms race over offspring sex [Hurst and Werren, 2001; Werren, 2011]. It has been suggested that such a conflict can drive the evolution of changes in host reproduction and sex determination mechanisms [Werren and Beukeboom, 1998; Stouthamer et al., 2010; Cordaux et al., 2011; Beukeboom, 2012]. Hence, these endosymbionts may be important evolutionary drivers of turnover in arthropod sex determination.

Here, we review and discuss the current knowledge about manipulative actions of endosymbionts in arthropods. We first briefly summarize the current knowledge about arthropod sex determination and the 4 major endosymbiotic manipulation types of host reproduction. We then move to a specific focus on how symbionts might interfere with host sex determination based on the current knowledge about the molecular basis of host manipulation. We end by proposing future directions on how these reproductive phenotypes may be key to further studies of arthropod sex determination. As epigenetic effects are becoming more apparent in insect development, we pay special attention to the possibility of epigenetic regulation.

Arthropod Sex Determination

Sex determination in arthropods is generally genetically determined by factors on sex chromosomes, with some exceptions in crustaceans in which it is under either temperature or photoperiod control [Bouchon et al., 1998; Cordaux et al., 2011; Kageyama et al., 2012]. Most knowledge comes from insects where sex determination occurs through a cascade of genes with a highly conserved master switch gene *(doublesex)* at the bottom but more divergence in the upstream genes (e.g. *transformer*) and the primary signals at the top of the cascade [Wilkins, 1995; Beye et al., 2003; Verhulst et al., 2010; Beukeboom, 2012]. The chromosomal constitutions serve as primary signals and vary between orders. Most insect orders (22 out of 29) have male heterogamety with either an XO or XY chromosomal constitution [Blackman, 1995; Beukeboom and Perrin, 2014]. For instance, most Diptera (flies) and Coleoptera (beetles) have male heterogamety with presence of a Y chromosome (XX/XY), and most Orthoptera (grasshoppers), Odonata (dragonflies), and Mantodea (mantids) have male heterogamety without a Y (XX/XO). All Lepidoptera (butterflies, moths) and Trichoptera (caddisflies) have female heterogamety (either ZW/ZZ or ZO/ZZ). Hymenoptera (sawflies, wasps, bees, and ants) and Thysanoptera (thrips) do not have specific sex chromosomes and reproduce by haplodiploidy (haploid males, diploid females). In addition to these common types of sex determination, more rare variations occur, such as multiple sex chromosomes and X chromosome or paternal genome loss [Bull, 1985; Sánchez, 2008].

The chromosomal constitutions are translated into different downstream signals that are also diverse among insect orders. In diploids they include X (or Z) chromosome counting elements, dominant masculinizing factors, and dominant feminizing factors. In haplodiploids, allelic complementarity at one or more sex determination loci and maternal effect genetic imprinting have been documented. In most species these signals converge downstream to regulate a key sex determination gene *transformer* which directly regulates the sex master switch gene *doublesex (dsx)* [Bull, 1985; Nöthiger and Steinmann-Zwicky, 1985; Wilkins, 1995; Marín and Baker, 1998; Raymond et al., 1998; Schütt and Nöthiger, 2000; Graham et al., 2002; Saccone et al., 2002; Sánchez, 2008; Verhulst et al., 2010; Gempe and Beye, 2011]. Exceptions seem to occur in Lepidoptera where *transformer* has not been found [Suzuki et al., 2001, 2008; Geuverink and Beukeboom, this issue]. *Doublesex* in turn regulates genes for sex specific development [Wilkins, 1995; Raymond et al., 1998; Schütt and Nöthiger, 2000] and together with the *fruitless* gene regulates sexual differentiation including sexual behavior [Waterbury et al., 1999; Rideout et al., 2010].

Much less is known about arthropod sex determination outside of the insects, in particular at the level of genes. In crustaceans, heterogametic sex determination appears to be most common [Legrand et al., 1987]. The *transformer* gene has been only identified in the water flea *Daphnia magna* but does not show sex differences in expression or splicing patterns, rendering it unlikely to be involved in sex determination [Kato et al., 2010]. An important difference from insects is that sex determination in crustaceans is an endocrine process mediated by the androgenic hormone synthesized by the androgenic gland [Ventura et al., 2011]. Basically, individuals have all

the genetic information to develop as male or female, but their fate is determined by a feminizing gene that inhibits the development of the androgenic gland and the synthesis of the androgenic hormone. In absence of the androgenic hormone, female differentiation is induced. In Acari (mites, ticks), both diploidy and haplodiploidy occur, but virtually nothing is known about the genetic regulation of sex determination [Norton et al., 1993; Arakaki et al., 2001]. The same holds for myriapods (millipedes, centipedes) that have male heterogametic sex determination [Fontanetti et al., 2002]. No sex determination genes have been identified in any of these arthropod groups yet.

Endosymbiont Diversity and Manipulation Types

Over 40% of all arthropods are infected with endosymbionts that live in the cytoplasm of their cells and are vertically transmitted through the eggs of females [Werren, 1997; Werren and O'Neill, 1997; Zchori-Fein et al., 2001; Zchori-Fein and Perlman, 2004; Zug and Hammerstein, 2012]. Some of these are obligate mutualists such as *Buchnera* in aphids [Douglas, 1998; Koga et al., 2003], but many others are reproductive parasites. The most prevalent of these host manipulators are the alpha-proteobacteria *Wolbachia pipientis* and *Rickettsia* sp., the bacteroidetes *Cardinium hertigii*, the gamma-proteobacterium *Arsenophonus,* and the mollicutes *Spiroplasma poulsonii* and *S. ixodetis* which belong to very distantly related bacterial clades [Duron et al., 2008]. Four broad categories of host reproductive manipulation are distinguished: induction of cytoplasmic incompatibility between egg and sperm, thelytokous parthenogenetic reproduction, killing of male offspring, and feminization of genotypic males [Hurst et al., 2002; Werren et al., 2008; Kraaijeveld et al., 2011]. The molecular genetic details of the mechanisms by which these endosymbionts exert the effects on their hosts are not yet well known. Given the diversity of effects and the variety of microorganisms involved, different questions arise: is this true convergence or are horizontal gene transfers between symbionts involved? If this is convergence among symbionts, is it only at the phenotypic level or also at the mechanistic level? How can we explain the seemingly easy evolution of these manipulations? Do different types of manipulation share common mechanisms? Answering these questions requires a better understanding of the molecular mechanisms at play which in turn will pave the way for better understanding the basic processes of sex determination and their evolution. Before getting into these questions, we briefly present the different types of reproductive manipulations. The common theme is that host sex determination is somehow manipulated by the endosymbionts to increase their own transmission vertically through females. Recent evidence suggests that some of these manipulative actions may be attained by directly interfering with host sex determination genes [Beukeboom, 2012; Sugimoto and Ishikawa, 2012].

Cytoplasmic Incompatibility

Cytoplasmic incompatibility (CI) is considered as the most widespread endosymbiotic manipulation among arthropods [Werren et al., 2008; Kageyama et al., 2012]. It has been found in Coleoptera, Diptera, Hymenoptera, Hemiptera, Lepidoptera, Orthoptera, Isopoda, Trombidiformes, and Mesostigmata [Tram and Sullivan, 2002; Werren et al., 2008; Kageyama et al., 2012] (table 1). Despite this broad phylogenetic distribution, CI induction has thus far only been attributed to *Wolbachia* and *Cardinium*. CI is a form of post-zygotic reproductive isolation occurring in crosses between infected males and uninfected females or when mates harbor different strains of the symbiont [O'Neill et al., 1992; Turelli and Hoffmann, 1995; Werren, 1997]. In diploid species, incompatible crosses produce severe cell cycle defects in the male-derived pronucleus, resulting in an abnormal chromosome condensation at metaphase and aberrant segregation during anaphase of the first mitotic division which leads to early embryonic mortality [Serbus et al., 2008]. In haplodiploids, CI crosses lead to male-biased offspring, because elimination of the paternal chromosome set restores haploidy and results in male development [Breeuwer and Werren, 1990, 1993; Breeuwer, 1997; Raychoudhury and Werren, 2012]. However, in some species haploid embryos may also die in an early stage, depending on the host species, genotype, or the symbiont complement [Vavre et al., 2000, 2001; Perrot-Minnot et al., 2002; Hunter et al., 2003; Mouton et al., 2005] due to the incomplete elimination of paternal chromosomes resulting in aneuploidy and thus unviable embryos [Tram et al., 2006]. The exact mode of action is not fully understood, but the current model is based on a chromosome marking effect during male gametogenesis that is rescued in the egg if endosymbionts (inherited from the mother via the egg cytoplasm) of a similar type are present [Werren et al., 2008]. CI thus results from a delayed paternal effect as *Wolbachia* or *Cardinium* are not present in the sperm. The sequencing of a CI-inducing *Cardinium* genome was expected to provide insights into the mechanisms of CI, but the recent publication of this genome did not yield

Table 1. Association between endosymbionts, arthropod host orders, and host sex determination (summarized from Kageyama et al. [2012])

Manipulation phenotype	Endosymbiont	Arthropod host order	Host sex determination (number of species reported)
Cytoplasmic incompatibility	*Wolbachia*	Coleoptera	XY or XO male heterogamety (7)
	Cardinium	Diptera	XY or XO male heterogamety (18)
		Hymenoptera	haplodiploidy (9)
		Hemiptera	XY male heterogamety (3)
		Lepidoptera	ZW or ZO female heterogamety (5)
		Orthoptera	XO or XY male heterogamety (6)
		Isopoda	ZW female heterogamety (2)
		Trombidiformes	haplodiploidy (6)
		Mesostigmata	unknown (1)
Parthenogenesis	*Wolbachia*	Hymenoptera	haplodiploidy (24)
	Cardinium	Thysanoptera	haplodiploidy (1)
	Rickettsia	Trombidiformes	haplodiploidy (2)
Male killing	*Wolbachia*	Coleoptera	XY male heterogamety (4), ZW female heterogamety (4), unknown (3)
	Spiroplasma		
	Rickettsia		
	Arsenophonus	Diptera	XY male heterogamety (14)
	Flavobacteria	Pseudoscorpiones	XO male heterogamety (1)
	Microsporidia parasites	Hemiptera	XO male heterogamety (1)
	unknown virus	Lepidoptera	ZW or ZO female heterogamety (13)
		Hymenoptera	haplodiploidy (1)
Feminization	*Wolbachia*	Lepidoptera	ZW or ZO female heterogamety (2)
	Cardinium	Hemiptera	XO male heterogamety (1)
	Microsporidia parasites	Hymenoptera	haplodiploidy (2)
	Gasteromermis	Trombidiformes	haplodiploidy (3)
	f factor (unknown)	Isopoda	ZW female heterogamety (2), XY male heterogamety (1), unknown (2)
		Ephemeroptera	unknown (1)
		Amphipoda	unknown (4)

more information. Interestingly though, it suggests that CI has an evolutionary independent origin in *Wolbachia* and *Cardinium*, since no recent horizontal gene transfer between these 2 symbionts has been detected [Penz et al., 2012]. CI-*Wolbachia* can readily spread in populations, because infected females have an advantage over uninfected females in that they are compatible both with uninfected and infected males [Werren, 1997].

Thelytokous Parthenogenesis

Several types of endosymbionts have been found to induce thelytokous parthenogenesis including *Wolbachia*, *Cardinium,* and *Rickettsia* [Werren, 1997, 2008; Giorgini et al., 2010] (table 1). Parthenogenesis induction (PI) by microbes entails making the host reproduction independent of fertilization. This results in progeny that consist entirely of females if the parthenogenesis induction is 100% effective. Parthenogenetic development of eggs requires special adaptations to the mode of oogenesis, i.e. the diploid complement needs to be restored after meiosis. There are many ways in which this could be accomplished [Suomalainen et al., 1987; Stenberg and Saura, 2009], including several modifications of meiosis, but the mechanisms used by endosymbionts appear rather limited (see below). Moreover, the taxonomic distribution of endosymbiont-induced thelytokous parthenogenesis in arthropods is quite restricted. Thus far, it has only been documented in haplodiploids, like hymenopterans, thrips, and mites (table 1). In these groups, the endosymbionts cause doubling of the chromosomes in the egg without subsequent cell division. Because of haplodiploid sex determination, the haploid eggs that would normally develop into males are converted into diploid eggs that develop into females [Werren et al., 2008]. In other words

the sex reversal is opposite to that of CI: genetic males are converted into genetic females by changing the chromosome complement of the zygote from haploidy to diploidy. Curing of hosts from their endosymbionts with antibiotics typically results in the production of haploid eggs that develop into males.

Cytological studies on a number of hymenopterans have revealed several different post-meiotic mechanisms of diploidy restoration. In *Trichogramma pretiosum, T. deion*, and *T. nr. deion*, diploidization is due to a segregation failure of the 2 sets of chromosomes in the first mitotic anaphase [Stouthamer and Kazmer, 1994]. A similar mechanism occurs in *Leptopilina clavipes* [Pannebakker et al., 2004]. In *Muscidifurax uniraptor*, however, the normal first mitotic anaphase is followed by fusion of the adjacent first mitotic nuclei [Gottlieb et al., 2002], a process known as gamete duplication. The result is 2 identical sets of chromosomes and completely homozygous progeny. In the mite *Bryobia praetiosa*, reproduction is functionally apomictic with all progeny identical in genotype to their mother and heterozygosity being maintained [Weeks and Breeuwer, 2001]. The similar functionally apomictic cloning mechanism was also found in the heterozygous offspring of the *Rickettsia*-infected parasitoid wasp *Neochrysocharis formosa* [Adachi-Hagimori et al., 2008].

PI is the ultimate strategy for a maternally transmitted symbiont: as fertilization is superfluous, fixation of the symbiont within populations or entire species is possible. Curing of hosts from their endosymbionts with antibiotics typically results in male production [e.g. Zchori-Fein et al., 2001; Kremer et al., 2009]. However, restoration of sexual lines has yet proved impossible in species in which the endosymbiont is fixed. Sexual traits have decayed either both in males and females, or males partially retain functionality. Two alternative explanations have been proposed. The neutral mutation hypothesis states that if traits involved in sexual reproduction are neutral under asexuality, relaxed selection might take place and allow mutations to accumulate, for instance, in male sexual traits such as courtship behavior and fertility. The selection hypothesis considers that sexual traits decay can be selected in females. First, if sexual traits are costly and no longer provide fitness benefits, they are expected to be strongly negatively selected. This applies more to female than to male sexual traits, like pheromone production, spermatheca functionality, and egg fertilization, because the males are absent under asexuality [Fong et al., 1995; Schwander et al., 2013]. Second, when *Wolbachia* infection remains polymorphic through inefficient transmission of the symbiont, a nucleo-cytoplasmic conflict over sex ratio may select nuclear alleles for higher male production, referred to as 'virginity mutants', which can be achieved by losing the ability to use sperm or losing the ability to mate [Stouthamer et al., 2010]. Whatever the mechanism at play, PI symbionts are associated with loss of traits involved in the normal process of sexual reproduction, and this process can be either neutral or actively selected for which opens up the possibility that endosymbionts take over the role of genes involved in sex determination and sexual differentiation.

Male Killing

Male killing (MK) is induced by a large diversity of endosymbiont taxa and found in a variety of arthropod host orders (table 1). *Wolbachia, Spiroplasma, Rickettsia*, *Arsenophonus*, *Flavobacteria*, as well as microsporidia have all been reported to cause male killing [reviewed in Hurst and Jiggins, 2000; Kageyama et al., 2012]. Male killing occurs if sons of infected mothers are killed by the endosymbiont during development [Bonte et al., 2008; Werren et al., 2008]. Endosymbiont-induced male lethality has been reported from 6 different arthropod orders, i.e. Coleoptera, Diptera, Pseudoscorpiones, Hemiptera, Lepidoptera, and Hymenoptera [Werren et al., 2008; Kageyama et al., 2012] (table 1). The MK phenotype is variable and can be divided into 2 broad categories according to the timing of action: early male killing at embryonic stages and late male killing at late larval or early pupal stages [Hurst, 1991; Kageyama et al., 2007]. Of interest, male killing is found in species with either male or female heterogamety as well as haplodiploidy which suggests, together with developmental timing variation, that male killing is the outcome of different molecular mechanisms (table 1; fig. 1). Early male killing is typically encountered in species where intra-brood competition is high; killing brothers allows sisters to have more resources for survival. Late male killing is associated with parasites having both vertical and horizontal transmission. The microorganisms gain the maximal benefit from it, because male hosts, which do not contribute to vertical transmission, are killed at the late larval stage when the number of infected cells is maximal allowing for the maximal horizontal transmission [Hurst, 1991; Kageyama et al., 2007; Nakanishi et al., 2008]. Importantly, the presence of male-killing selfish elements leads to selection for host resistance. This is notably what occurred in the butterfly *Hypolimnas bolina* where Asian populations harbor a dominant resistant allele to the male-killing phenotype, although the mechanistic details are not known yet [Hornett et al.,

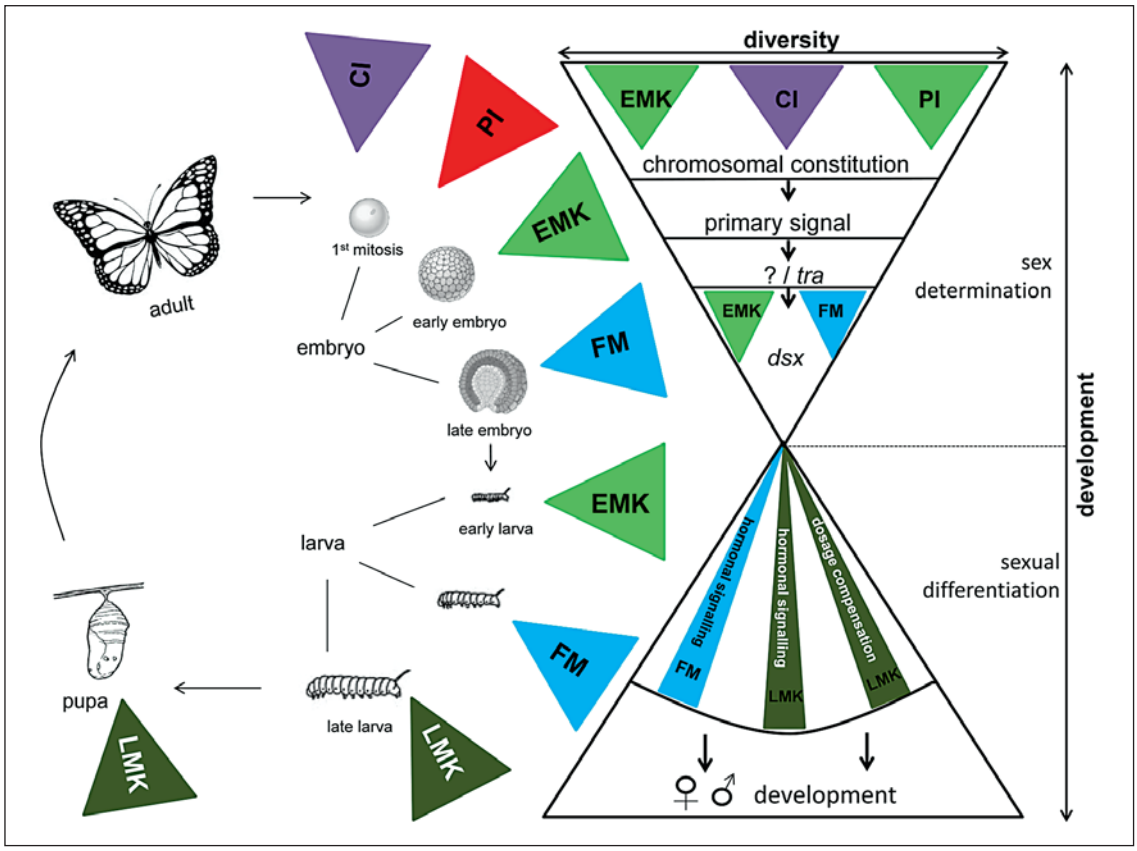

Fig. 1. The 4 manipulation phenotypes of endosymbionts that affect different developmental stages of arthropods (using a butterfly life cycle as an example). Red arrow: thelytokous parthenogenesis induction (PI); purple arrows: cytoplasmic incompatibility (CI), blue arrows: feminization (FM); light green arrows: early male killing (EMK), and dark green arrows: late male killing (LMK) in terms of the developmental stage at which MK occurs. Each arrow indicates the corresponding host developmental stage at which endosymbiotic manipulation takes place. The sex determination-differentiation pathway is enlarged to depict the position in the gene cascade and timing during development at which endosymbionts interfere. *Transformer (tra)* is the central gear to transmit the primary signals to the conserved master switch gene *doublesex (dsx)* which regulates the downstream sexual differentiation. The question mark next to *tra* refers to insects in which this gene appears to be absent, such as Lepidoptera.

2008]. Interestingly, the rapid spread of resistance has been monitored in natural populations of the South Pacific, highlighting both the dynamic nature of these interactions and the intensity of the selective pressures generated by reproductive manipulators [Charlat et al., 2007].

Feminization

Conversion of genotypic males into phenotypic and functional females is known as feminization (FM) [Bouchon et al., 1998; Kageyama et al., 1998]. Endosymbiont-induced feminization has been reported from 7 arthropod orders: Lepidoptera, Hemiptera, Hymenoptera, Thrombidiformes, Isopoda, Ephemeroptera, and Amphipoda [reviewed in Werren et al., 2008; Kageyama et al., 2012]. Feminization is associated with different sex determination mechanisms in these groups, such as male or female heterogamety, haplodiploidy, and some unknown mechanisms for crustacean species (table 1). Feminization seems to be more frequent in crustaceans than in insects which could be due to the easiness to manipulate sexual phenotypes in the former. Indeed, simple manipulation of hormonal levels in crustaceans leads to sex reversion. In the well-studied woodlouse *Armadillidium vulgare* (Isopoda), *Wolbachia* feminizes ZZ males by interfering with the production/perception of the androgenic hormone from the male developmental gland during sexual differentiation [Bouchon et al., 2008; Cordaux et al., 2011]. This resembles the shrimp *Gammarus duebeni* in which microsporidian parasites such as *Octosporea effeminans* and *Nosema granulosis* change males into function-

al females [Bulnheim and Vávra, 1968; Terry et al., 1998; Rodgers-Gray et al., 2004]. Feminization has also been found in insects where different mechanisms could be at play such as disrupting methylation patterns and genetic imprinting in the male-heterogametic leafhopper *Zyginidia pullula* [Negri et al., 2006, 2009] or altering splicing of *doublesex* in the female-heterogametic butterfly *Eurema mandarina* [Narita et al., 2007]. Feminization also occurs in haplodiploid species. Giorgini et al. [2009] found that in *Encarsia hispida* curing from *Cardinium* does not lead to haploid but diploid males, suggesting that the endosymbionts are not responsible for genome duplication (parthenogenesis) but rather cause feminization of diploid males. Moreover, in the *Cardinium* infected mite *Brevipalpis phoenicis* [Groot and Breeuwer, 2006], consisting exclusively of haploid females, Weeks et al. [2001] reported that curing of the bacterium changes haploid daughters into haploid sons.

Under endosymbiont-induced feminization, scarcity of males within host populations generates a strong nucleo-cytoplasmic conflict. Resistance forms have been detected in some cases, notably in *A. vulgare*. In this species, together with masculinizing genes, other feminizing factors have been demonstrated but are encoded by the nuclear genome [Juchault and Mocquard, 1993]. There is some evidence that this nuclear feminizing factor originates from a horizontal gene transfer from *Wolbachia* [Rigaud and Juchault, 1995]. The *A. vulgare* system is a good illustration of the dynamic nature of sex determination where female and male heterogamety are evolving in response to feminizing *Wolbachia* [Cordaux et al., 2011]. The high diversity and dynamics of sex determination systems and the absence of sex chromosome differentiation in crustaceans makes it likely that this pattern occurs more widespread in crustaceans [Rigaud, 1997].

Mechanisms of Reproductive and Sex Determination Manipulations

With respect to genetic mechanisms, we delineate a typology of reproductive manipulations. CI, PI, MK, and FM differ in their actions in relation to the timing at which they interfere with the host sex determination and differentiation processes. Taking the master sex switch gene *doublesex* as the central point ('bottleneck in an hourglass'), manipulations can target earlier events constituting the primary signals, *doublesex* itself, or downstream processes including sexual differentiation (fig. 1). This typology integrates phylogenetic and empirical information and allows us to consider different reproductive manipulation mechanisms in a phylogenetic context. It indicates that endosymbionts have the potential to undergo rapid evolutionary shifts in phenotypes [Werren, 1997; Jaenike, 2007; Kraaijeveld et al., 2011]. Below, we discuss how mechanisms of reproductive manipulation may be informative for the molecular basis of host sex determination.

Interference with Primary Signals

Interference with primary sex determination signals concerns notably manipulation of chromosomal behavior. This is clearly established for CI where paternal effects lead to ploidy changes in the early fertilized egg. CI endosymbionts in diploid arthropod species obviously do not interfere with host sex determination, because they cause lethality through haploidization of eggs [Serbus et al., 2008]. However, in haplodiploids, conversion of diploid female eggs into haploid male eggs occurs by changing the zygotic chromosomal constitution that acts as a primary signal for sex determination. This is very similar to PI endosymbionts that also act early during sex determination as they alter the number of chromosomal complements at the end of the first or beginning of the second mitotic division. As transcription is probably limited at that time, PI is certainly a parental effect, but contrary to CI, it is limited to a maternal effect. It is still unknown how endosymbionts precisely alter the molecular regulation of mitosis to induce diploidization of the host eggs. Why parthenogenesis inducing microbes have not been found in diploid species remains another mystery. One explanation is that PI evolves more easily in haplodiploids because of the pre-existing cellular machinery for full development from unfertilized haploid eggs. The interactions between mechanisms of sex determination and PI endosymbionts are particularly complex and further elaborated in the section "Interaction between PI Endosymbionts and Host Haplodiploid Sex Determination". The early MK type can also act on the zygotic chromosome constitution that serves as the primary signal in the host sex determination pathway. In the wasp *Nasonia vitripennis*, *Arsenophonus nasoniae* kills male offspring by blocking maternal centrosome formation during oogenesis [Ferree et al., 2008]. In *Drosophila bifasciata*, infected male embryos show severe defects of chromatin remodeling and spindle organization, a phenotype strikingly similar to the phenotype observed in CI [Riparbelli et al., 2012].

Early acting endosymbionts that alter the chromosomal constitution, a feature of PI, CI, and early MK, suggest similar target host genes that have a relatively broad

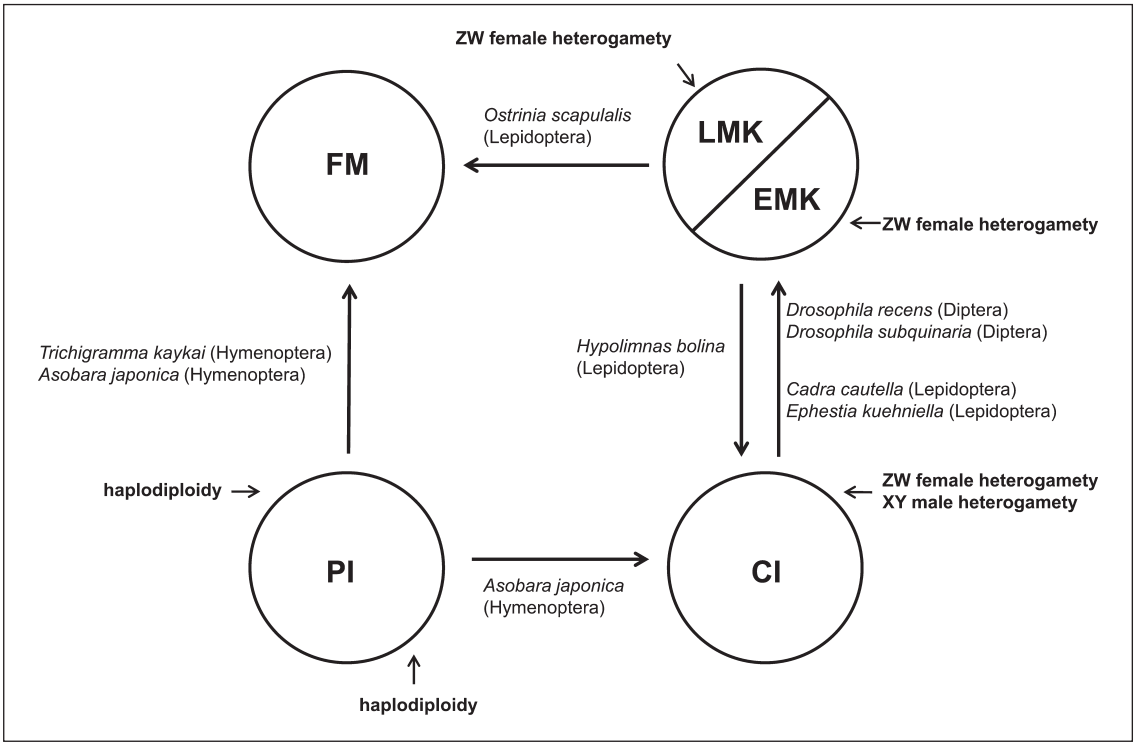

Fig. 2. Transitions between the 4 different manipulative phenotypes of *Wolbachia*. FM = Feminization; EMK = early male killing; LMK = late male killing; CI = cytoplasmic incompatibility; PI = thelytokous parthenogenesis induction. The reported species (and orders) are indicated at each arrow as well as their mode of sex determination.

function. This would explain why the manipulations occur in such a diversity of host taxa regardless of their sex determination system. There are many molecules that endosymbionts could target to change the chromosome constitution of the egg. Of particular interest, CI is associated with impaired histone deposition in the male pronucleus which could lead to activation of cell cycle checkpoints [Landmann et al., 2009]. Other examples include the inhibition of the proper digestion of cohesions that would result in failure of chromosome separation during meiosis or mitosis [Ferree et al., 2008; Schurko et al., 2009]. A similar effect might be achieved by interfering with signals that regulate the M checkpoint in the cell cycle. An interesting class of potential target genes are meiosis related genes which code for Argonaute proteins or mitotic division related genes coding for cell cycle proteins [Schurko et al., 2009; Kraaijeveld and Bast, 2012]. Informatively, *Wolbachia*-induced CI can transit to MK (fig. 2) as was found in 2 *Drosophila* and 2 moth species. MK occurred when uninfected males of *Drosophila subquinaria* mated with hybrid females from the cross between *Drosophila recens* females with the CI phenotype and endosymbiont-uninfected *D. subquinaria* males [Sasaki et al., 2002, 2005; Jaenike, 2007]. Interestingly, the same transition but in opposite direction from MK to CI occurred in the butterfly *Hypolimnas bolina* [Hornett et al., 2008]. The suppression of the MK phenotype in infected individuals resulted in male production which upon mating with uninfected females induced CI (fig. 2). These studies suggest that it is relatively easy to shift between MK and CI and point towards similar mechanisms. Transitions can also occur from PI to CI. In *Asobara japonica*, male offspring produced by PI-*Wolbachia*-infected females induced (moderate) CI against uninfected females [Kraaijeveld et al., 2011] (fig. 2).

Direct Interference with Doublesex

Late acting endosymbionts are associated with sexual differentiation and must recognize maleness resulting from male-specifically expressed genes during development. It is now evident that endosymbionts can directly interfere with the expression of sex determination genes. For example, male killing in the moth *Ostrinia scapulalis* is accomplished by altering the splicing of *doublesex* [Su-

gimoto and Ishikawa, 2012]. Altered splicing is also found in the butterfly *Eurema mandarina* in which *Wolbachia*-infected genetic males (ZZ) are morphologically and behaviorally fully female and completely fertile. The splicing pattern of the sex-determining gene *dsx* changes according to the *Wolbachia* infection status. Intersex individuals express both female and male *dsx* splice variants. The lethal effects normally occur during late embryonic or early larval developmental stages and might be due to disruption of dosage compensation [Kageyama and Traut, 2004; Narita et al., 2007; Sakamoto et al., 2007; Sugimoto et al., 2010; Sugimoto and Ishikawa, 2012]. It is still unknown whether *Wolbachia* directly acts on *dsx* splicing or (more probably) on an upstream splicing regulator of *dsx* in this female heterogametic system [Beukeboom, 2012]. In the *Spiroplasma* infected ladybird beetle *Anisosticta novemdecimpunctata*, males are killed in the early embryonic stage [Tinsley and Majerus, 2006], but the genetic mechanism is still unknown as is true for all MK types in ladybirds [Balayeva et al., 1995; Hurst et al., 1996]. These examples of early MK show that the microbes have evolved different ways of killing males. The MK in *Ostrinia* is the first well documented case of direct interference of endosymbionts upon host sex determination genes. Due to being the central gear of the key sex determination gene, *transformer* is expected to be a particularly likely target for such manipulation in holometabolous insect sex determination [Beukeboom, 2012; Negri and Pellecchia, 2012].

Interference during Sexual Differentiation

Male killing can also occur in the sexual differentiation phase of embryonic or larval development. A functional dosage-compensation complex, a major component of sexual differentiation in *Drosophila melanogaster*, is required for male killing by *Spiroplasma*. *Spiroplasma* failed to kill males lacking any of the 5 proteins required for proper dosage compensation [Veneti et al., 2005]. Dosage compensation is tightly connected with sex determination in *Drosophila* as the gene *sex lethal*, which has both a function in dosage compensation and in sex determination, acts as a splice regulator of *transformer* [Cline, 1984]. Although yet speculative, it may be that the MK *Spiroplasma* targets the *sex lethal* gene [Starr and Cline, 2002]. In the mosquito *Aedes stimulans*, *Amblyospora* microsporidia kill males in the fourth larval stage [Andreadis, 1985] which is another example of late male killing. Furthermore, an unknown RNA virus was found responsible for late male killing in the oriental tea tortrix, *Homona magnanima,* in which male death occurs in the larval or pupal stage [Nakanishi et al., 2008].

Hormonal signaling pathways are frequently involved in the regulation of symbiotic interactions. In parasitic interactions such as host-parasitoid relationships, they play a central role in synchronizing host and parasite cycles, and manipulation of hormonal signaling by each party has been found [Sagi and Khalaila, 2001; Negri, 2011; Jahnke et al., 2013]. Hormonal signaling as a part of sexual differentiation can also be manipulated by endosymbionts. This is apparent in crustaceans where the establishment of the sexes is a hormonal process. Notably, injection of *Wolbachia* in young males of *A. vulgare* induces hypertrophy of the androgenic gland and feminization of tissues [Rigaud and Juchault, 1995]. This result indicates that *Wolbachia* may interfere with the androgenic hormone receptors and either antagonize the fixation of the androgenic hormone on these receptors or decrease their production. The androgenic hormone is related to insulin and/or insulin-like growth factors which is interesting for 2 reasons. First, *Wolbachia* has been shown to interact with the insulin pathway in *Drosophila* [Ikeya et al., 2009]. Even though this pathway is not directly involved in sex determination, insulin-like peptides regulate ecdysteroid synthesis, and recent results indicate that 20-Hydroxyecdysone could play the role of a sex hormone in insects [Negri et al., 2010; Negri and Pellecchia, 2012]. Hormonal manipulation seems mostly associated with feminization, but male killing may also make use of hormonal signals that are different between the sexes. It should, however, be noted that sex determination in insects is generally considered as a cellular genetic process and that the importance of hormonal signaling is still under debate [Steinmann-Zwicky et al., 1989; Schütt and Nöthiger, 2000; Negri and Pellecchia, 2012]. This is informative for the transition between MK and FM which is observed in the moth *Ostrinia scapulalis*. Antibiotic treatment induced intersex individuals, suggesting that MK-inducing *Wolbachia* were also responsible for feminization [Kageyama and Traut, 2004; Sakamoto et al., 2008; Sugimoto and Ishikawa, 2012]. In addition, transition from PI to FM occurred in the parasitoid wasp *Trichogramma kaykai*. In *T. kaykai* with a PI phenotype, diploid intersex individuals were produced under high temperature, suggesting that PI-*Wolbachia* are also responsible for feminization that is dependent on *Wolbachia* density [Tulgetske and Stouthamer, 2012] (fig. 2). A small proportion of diploid males is also regularly detected in the parasitoid wasp *Asobara japonica* which suggests that PI-*Wolbachia* are required for feminization and that this effect is dependent on *Wolbachia* density [W.-J. Ma, unpubl. data].

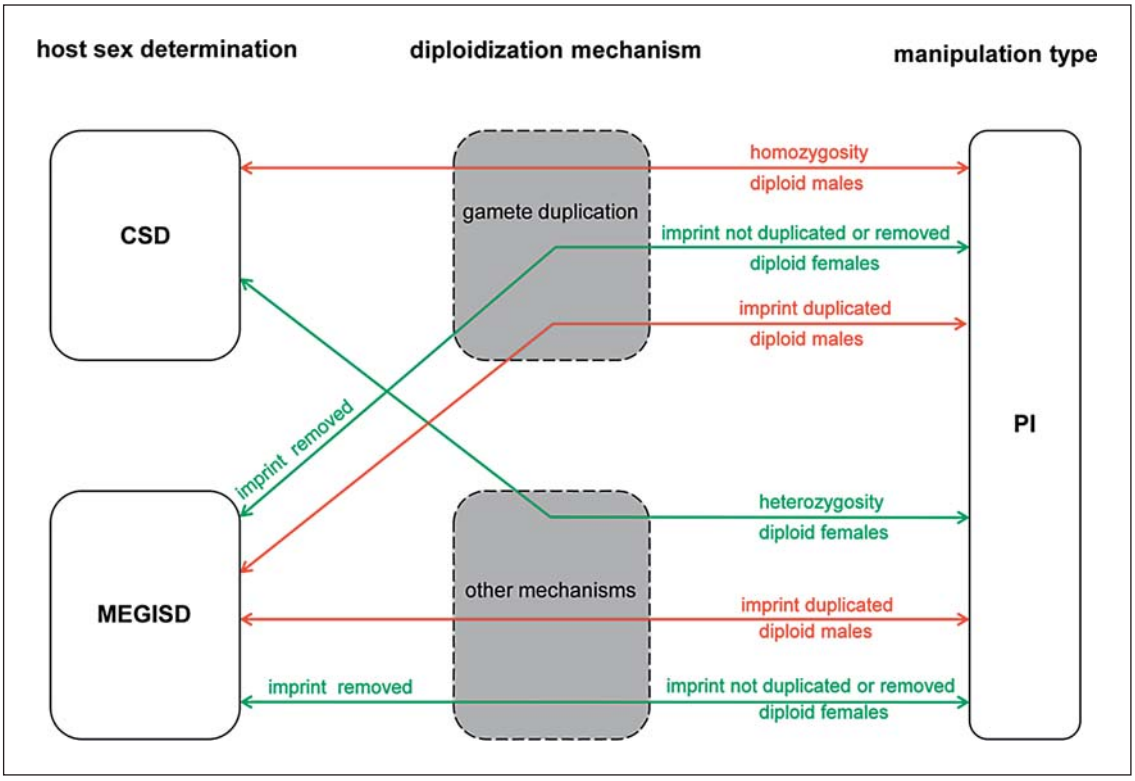

Fig. 3. PI-inducing endosymbionts and haplodiploid host sex determination. CSD = Complementary sex determination; MEGISD = maternal effect genomic imprinting sex determination. Red arrows: incompatible combinations; green arrows: compatible combinations. CSD is only compatible with PI if diploidization is other than by gamete duplication (e.g. premeiotic doubling, central or terminal fusion). MEGISD species can only have PI if the maternal imprint that prevents female development is not copied during gamete duplication or the endosymbionts remove it before or after diploidization. The mode of sex determination and diploidization are mutually informative on each other: in *Lepidoptera clavipes* and *Trichagramma kaykai*, PI occurs by gamete duplication and CSD is excluded as sex determination [Schilthuizen et al., 1998; Pannebakker et al., 2004; Tulgetske, 2010], in *Asobara japonica* CSD is absent and diploidization could occur via gamete duplication [Kremer et al., 2009; Ma et al., 2013], and in *Encarsia hispida* feminization of diploid males can occur under MEGISD if *Cardinium* removes the imprint [Giorgini et al., 2009].

Interaction between PI Endosymbionts and Host Haplodiploid Sex Determination

The interaction between endosymbionts and haplodiploid host sex determination is complex, because the mechanisms by which diploidization of the egg takes place also affects the outcome. In some cases it dictates whether particular endosymbionts can establish a certain host phenotype (fig. 3). Several hymenopteran groups have complementary sex determination (CSD) in which sex is determined by the allelic composition of the sex locus: heterozygotes develop into females, hemizygotes and homozygotes into males [Whiting, 1933; Cook, 1993a; Beye et al., 2003]. CSD and PI-inducing endosymbionts that cause gamete duplication are incompatible [Cook, 1993b; van Wilgenburg et al., 2006], because this form of diploidization results in complete homozygosity in most documented species so far [Stouthamer and Kazmer, 1994; Pannebakker et al., 2004; Gottlieb et al., 2002]. The reason is that under CSD diploid homozygotes develop into males, whereas female development is required for PI endosymbionts to invade a host. There is indeed a phylogenetic association between the absence of CSD and the presence of PI endosymbionts [Heimpel and de Boer, 2008]. Interestingly, some CSD species do reproduce parthenogenetically, such as *Venturia canescens*, but in those species the diploidization mechanism is different (e.g. central or terminal fusion) and apparently retains sex locus heterozygosity [Suomalainen et al., 1987; Beukeboom and Pijnacker, 2000; Mateo-Leach et al., 2009]. Functionally apomictic cloning mechanism is also the case for the

mite *B. praetiosa* and the parasitoid wasp *N. formosa* [Weeks and Breeuwer, 2001; Adachi-Hagimori et al., 2008].

The other known genetic mechanism of sex determination in Hymenoptera is maternal effect genomic imprinting sex determination (MEGISD). Under MEGISD female development requires a paternal genome for activation of the *transformer* gene in the zygote which is silenced on the maternal complement [Verhulst et al., 2010; see also Verhulst and van de Zande in this issue]. It has thus far only been documented for *Nasonia vitripennis* (Chalcidoidea). The broader phylogenetic distribution of the MEGISD model has been challenged, because it is difficult to reconcile with parthenogenetic female reproduction in which a non-imprinted male genome is missing in the egg. One solution would be that the maternally provided imprint is not copied onto the duplicated genome during the diploidization process, providing an active *transformer* copy to the zygote without fertilization. Under this assumption, PI endosymbionts would be able to infect species with MEGISD. On the other hand, if the maternal imprint would be passed on, zygotic diploidy would result in males and PI by endosymbionts cannot be established (fig. 3). For other forms of diploidization, such as central and terminal fusion, it is necessary to assume that the endosymbionts can remove the maternal imprint, because fusion of 2 meiotic nuclei, each with a maternal imprint, would lead to diploid males. Only gamete duplication without imprint copying alleviates the requirement of endosymbiont interference with MEGISD (fig. 3). Further information is needed on the phylogenetic distribution of the MEGISD system before these issues can be solved.

In the chalcidoid *E. hispida*, diploid males are produced when females are cured from *Cardinium*. The type of endosymbiont action, following the above rationale, is thus informative for the sex determination mechanism of this species: it may have MEGISD without the maternal imprint copy (fig. 3, green lines originating from MEGISD). Taking the opposite argumentation, having MEGISD may have prevented it from being infected by PI endosymbionts (fig. 3, red lines originating from MEGISD). How egg diploidization and feminization occurs in this system is not yet known. Removal of the bacteria yields diploid males, indicating that egg diploidy is controlled by the host genotype. Assuming MEGISD, one possibility is that *Cardinium* prevents transmission of the maternal imprint to the duplicated genome copy, turning diploid male eggs into diploid female eggs.

Conclusion and Future Directions

Although there have been several reviews on endosymbiotic manipulation of arthropod host reproduction, we have taken a specific focus on the mechanisms by which endosymbionts may interfere with host sex determination. From considering the 4 major endosymbiotic manipulation types, it is clear that diverse endosymbionts can target the host at different developmental stages, ranging from the spermatogenesis stage or the first mitotic division to the late pupal stage. The evolution of similar manipulation types in distantly related endosymbiont taxa shows that convergent evolution has probably occurred repeatedly. Many of the intricacies of endosymbiont-host interactions remain to be discovered, because in most instances it is still unknown what developmental pathways are exploited by the endosymbionts to exert their effects on host reproduction. We have proposed that the transitions between endosymbiont phenotypes suggest partly similar mechanisms for apparently divergent phenotypes. We have also argued that the mechanism of endosymbiotic manipulation must be considered in the context of the host sex determination mechanism and that both of these processes may be mutually informative on each other.

With the development of next-generation DNA sequencing techniques, it is getting easier to acquire genomic information on non-model organisms which makes the unraveling of the genetic basis of endosymbiotic manipulation very promising and exciting. A first question to answer is whether the diversity of effects and the variation of microorganisms involved reflect true convergence or merely horizontal gene transfer between symbionts. Future studies should compare different endosymbiont genomes for gene composition as well as gene products that might affect developmental pathways of their hosts [e.g. Moreno et al., 2011]. For instance, the comparison of genomes between *Wolbachia* and *Cardinium* suggests that CI has an evolutionary independent origin in these 2 symbionts and reveals no evidence for recent horizontal gene transfer [Penz et al., 2012]. Comparison of transcriptomes and proteomes of infected and uninfected hosts may also be rewarding [e.g. McNulty et al., 2012]. Moreover, the integration of knowledge about evolutionary dynamics and genomic data should make it possible to identify genomic signatures that can lead to the identification of genes involved in host reproduction and sex determination manipulation. Attention should be paid to host sex determination genes such as *transformer* and *doublesex*, candidate targets for disruption by

endosymbionts, whose regulation may be altered in several ways, including their sex-specific splicing or imprinting. In addition, cell cycle genes involved in meiosis and mitosis, particularly those related to histone regulation or genes coding for Argonaute proteins, are good candidates [Kraaijeveld and Bast, 2012].

There is growing evidence for epigenetic control of developmental processes in insects [Lyko and Maleszka, 2011] as well as in host-parasite interactions [Gómez-Díaz et al., 2012]. Given the evolution of multiple reproductive manipulations, it is tempting to propose that these phenotypes may actually be mechanistically very close to other physiological mechanisms involved in host-parasite interactions that could represent pre-adaptations to reproductive manipulations [Vavre et al., 2003]. As reproductive manipulations often involve parental effects, epigenetic manipulation by endosymbionts clearly requires attention. An obvious candidate is chromatin remodeling which can lead to alteration of chromosomal behavior as well as to variation in gene expression or splicing processes. It is thus possible that many of the symbiont phenotypes rely on epigenetic mechanisms, particularly those related to histone regulation. Moreover, paternal effects of CI, maternal effects of PI, and mechanisms of early MK may all involve some form of genomic imprinting [Werren, 2011; Negri and Pellecchia, 2012; Rabeling and Kronauer, 2013]. The currently strongest evidence for a role of epigenetics was found by Negri et al. [2009] who showed that *Wolbachia* interferes with host sexual differentiation in the leafhopper *Z. pullula* by disrupting methylation patterns and genetic imprinting. In *Drosophila* species, *Wolbachia* prophage DNA adenine methyltransferase genes might be involved in the modification or rescue process of CI [Saridaki et al., 2011]. These studies are first indications for a role of epigenetics in host manipulation, but we are only at the beginning of elucidating the precise molecular and biochemical pathways involved. Technological developments now allow for easier characterization of epigenetic marks, and transcriptome and proteome comparison of infected and uninfected individuals in various systems may be a promising way forward. Without doubt, more mechanistic studies of host reproduction manipulation are going to reveal novel and intriguing insights into the co-evolution between host and endosymbiont reproduction.

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