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

W.-J. Ma[a]   F. Vavre[b]   L.W. Beukeboom[a]

[a]Evolutionary Genetics, Centre for Ecological and Evolutionary Studies, University of Groningen, Groningen, The Netherlands; [b]Laboratoire de Biométrie et Biologie Evolutive, Université de Lyon, Villeurbanne, France

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 haplo-

diploidy. Future directions on how the study of endosymbiotic manipulation of host reproduction can be key to further studies of arthropod sex determination are shown.

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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

Wen-Juan Ma
Evolutionary Genetics, Centre for Ecological and Evolutionary Studies
University of Groningen, PO Box 11103
NL-9700 CC Groningen (The Netherlands)
E-Mail wenjuanma84@gmail.com

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 Cardinium	Coleoptera	XY or XO male heterogamety (7)
		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 Cardinium Rickettsia	Hymenoptera Thysanoptera Trombidiformes	haplodiploidy (24) haplodiploidy (1) haplodiploidy (2)
Male killing	Wolbachia Spiroplasma Rickettsia Arsenophonus Flavobacteria Microsporidia parasites unknown virus	Coleoptera Diptera Pseudoscorpiones Hemiptera Lepidoptera Hymenoptera	XY male heterogamety (4), ZW female heterogamety (4), unknown (3) XY male heterogamety (14) XO male heterogamety (1) XO male heterogamety (1) ZW or ZO female heterogamety (13) haplodiploidy (1)
Feminization	Wolbachia Cardinium Microsporidia parasites Gusteromermis F factor (unknown)	Lepidoptera Hemiptera Hymenoptera Trombidiformes Isopoda Ephemeroptera Amphipoda	ZW or ZO female heterogamety (2) XO male heterogamety (1) haplodiploidy (2) haplodiploidy (3) ZW female heterogamety (2), XY male heterogamety (1), unknown (2) unknown (1) 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

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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 transmis-

sion 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.,
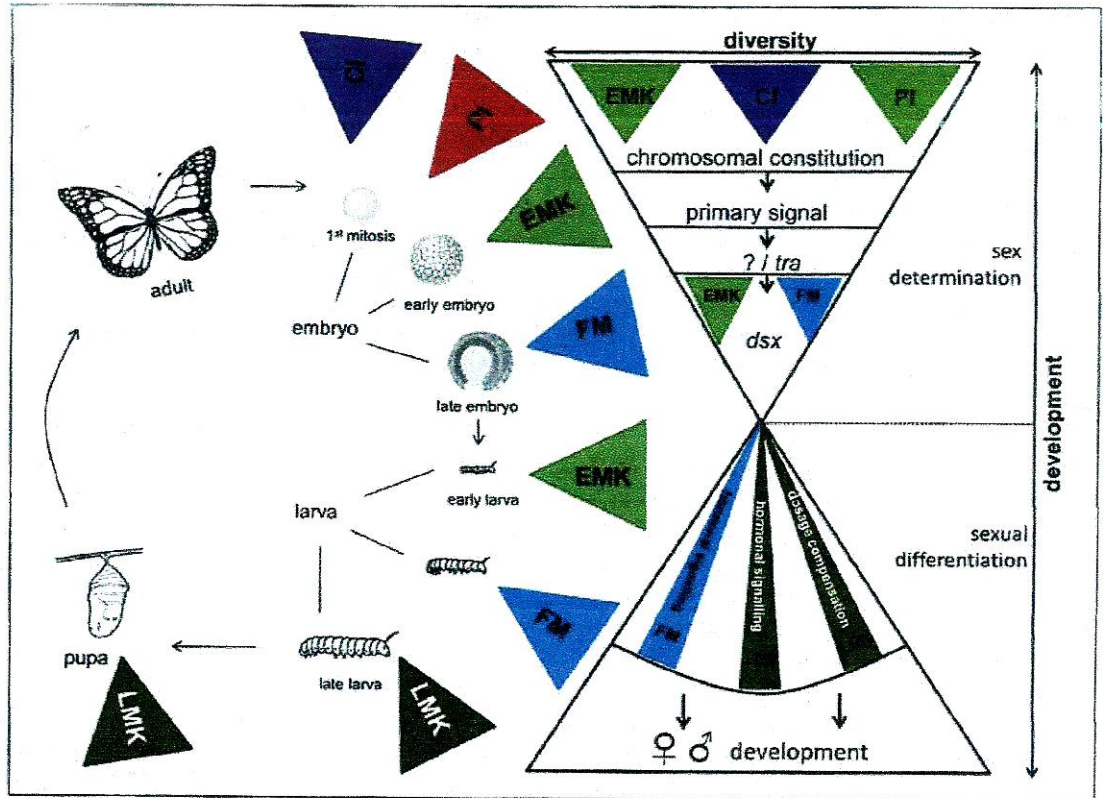
Fig. 1. The 4 manipulation phenotypes of endosymbionts that affect different developmental stages of arthropods (using a butterfly life cycle as an example). Red arrow: thelytokous parthenogenesis induction (PI); purple arrows: cytoplasmic incompatibility (CI), blue arrows: feminization (FM); light green 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 en- dosymbiotic 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 deter-

mination 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].