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1 **Manipulation of arthropod sex determination by endosymbionts: diversity and**
2 **molecular mechanisms**

3

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23 **Running title:** Endosymbiont Manipulation of Arthropod Sex Determination

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25

26 **Abstract**

27 Arthropods exhibit a large variety of sex-determination systems both at the chromosomal and
28 molecular level. Male heterogamety, female heterogamety, and haplodiploidy occur
29 frequently, but partially different genes are involved. Endosymbionts, such as *Wolbachia*,
30 *Cardinium*, *Rickettsia* and *Spiroplasma*, can manipulate host reproduction and sex
31 determination. Four major reproductive manipulation types are distinguished: cytoplasmic
32 incompatibility, thelytokous parthenogenesis, male killing and feminization. In this review, we
33 summarize the effects of these manipulation types, and how they interfere with arthropod sex
34 determination in terms of host developmental timing, alteration of sex determination and
35 modification of sexual differentiation pathways. Transitions between different manipulation
36 types occur frequently, which suggests that they are based on similar molecular processes. We
37 discuss how mechanisms of reproductive manipulation and host sex determination can be
38 informative on each other, with a special focus on haplodiploidy. We end with future
39 directions on how study of endosymbiont manipulation of host reproduction can be key to
40 further study of arthropod sex determination.

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46 **Key Words:** endosymbiont, arthropods, molecular mechanism, sex determination, sexual
47 differentiation, hormonal signaling, epigenetics

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54 **Introduction**

55 Arthropods cover over 1,2 million described species that account for about 80% of all
56 known living animal species. They have colonized virtually all habitats on Earth. In line with
57 this broad adaptation to many conditions they exhibit an enormous variety of life histories and
58 reproductive modes. They also show surprisingly large variation and turnover in sex-
59 determination systems. It is therefore a prime group of organisms to study how changes in
60 sex-determination mechanism come about, a current topic in evolutionary biology that is not
61 well understood. A particular aspect of arthropod biology is their frequent infection with
62 microorganisms that can be mutualistic, parasitic or commensal. A specific group are
63 endosymbionts, such as *Wolbachia*, *Cardinium*, *Rickettsia*, *Spiroplasma* and *Arsenophonus*
64 bacteria, microsporidia and viruses, that manipulate their host's reproduction in a variety of
65 ways [reviewed in Hurst et al., 1996; Werren et al., 2008; Kageyama et al., 2012]. These
66 intracellular parasites are maternally transmitted through the egg cytoplasm. As males are an
67 evolutionary dead end for them, any symbiont having the capability to increase female
68 production is at an advantage and can invade host populations [Partridge and Hurst, 1998;
69 Duron et al., 2008; Werren et al., 2008; Cordaux et al., 2011]. This can be realized through
70 causing thelytokous parthenogenesis, male killing or feminization. As they enhance their own
71 transmission at the expense of their host's fitness, their presence generates genetic conflicts
72 between the two sexes, and possibly an ensuing coevolutionary arms race over offspring sex

73 [Hurst and Werren, 2001; Werren, 2011]. It has been suggested that such conflict can drive the
74 evolution of changes in host reproduction and sex-determination mechanisms [Werren and
75 Beukeboom, 1998; Stouthamer et al., 2010; Cordaux et al., 2011; Beukeboom, 2012]. Hence,
76 these endosymbionts may be important evolutionary drivers of turnovers in arthropod sex
77 determination.

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

87

88

89 **Arthropod sex determination**

90 Sex determination in arthropods is generally genetically determined by factors on sex
91 chromosomes, with some exceptions in crustaceans in which it is under either temperature or
92 photoperiod control [Bouchon et al., 1998; Cordaux et al., 2011; Kageyama et al., 2012]. Most
93 knowledge comes from insects, where sex determination occurs through a cascade of genes
94 with a highly conserved master switch gene (*doublesex*) at the bottom, but more divergence in
95 the upstream genes (e.g. *transformer*) and the primary signals at the top of the cascade
96 [Wilkins, 1995; Beye et al., 2003; Verhulst et al., 2010; Beukeboom, 2012]. The chromosomal
97 constitutions serve as primary signals and vary between orders. In most insect orders (22 out
98 of 29), the chromosomal constitutions are either XO or XY male heterogamety [Blackman,
99 1995; Beukeboom and Perrin, 2014]. For instance, most Diptera (flies) and Coleoptera (beetles)
100 have male heterogamety with presence of a Y chromosome (XX/XY), and most Orthoptera

101 (grasshoppers), Odonata (dragonflies), and Mantodea (mantids) have male heterogamety
102 without a Y (XX/XO). All Lepidoptera (butterflies, moths) and Trichoptera (caddisflies) have
103 female heterogamety (either ZW/ZZ or ZO/ZZ). Hymenoptera (sawflies, wasps, bees and ants)
104 and Thysanoptera (thrips) do not have specific sex chromosomes and reproduce by
105 haplodiploidy (haploid males, diploid females). In addition to these common types of sex
106 determination, more rare variations occur, such as multiple sex chromosomes and X-
107 chromosome or paternal genome loss [Bull, 1985; Sánchez, 2008].

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

122 Much less is known about arthropod sex determination outside of the insects, in particular
123 at the level of genes. In crustaceans, heterogametic sex determination appears to be most
124 common [Legrand *et al.*, 1987]. The *transformer* gene has been only identified in the water
125 flea *Daphnia magna*, but does not show sex differences in expression or splicing patterns,
126 rendering it unlikely to be involved in sex determination [Kato *et al.*, 2010]. An important
127 difference from insects is that sex determination in crustaceans is an endocrine process
128 mediated by the androgenic hormone synthesized by the androgenic gland [Ventura *et al.*,
129 2011]. Basically, individuals have all the genetic information to develop as male or female, but

130 their fate is determined by a feminizing gene that inhibits the development of the androgenic
131 gland and the synthesis of the androgenic hormone. In absence of the androgenic hormone,
132 female differentiation is induced. In Acari (mites, ticks), both diploidy and haplodiploidy occur,
133 but virtually nothing is known about the genetic regulation of sex determination [Norton et al.
134 1993; Arakaki et al., 2001]. The same holds for myriapods (millipedes, centipedes) that have
135 male heterogametic sex determination [Fontanetti et al., 2002]. No sex-determination genes
136 have been identified in any of these arthropod groups yet.

137

138

139 **Endosymbiont diversity and manipulation types**

140 Over 40% of all arthropods are infected with endosymbionts that live in the cytoplasm of
141 their cells and are vertically transmitted through eggs of females [Werren, 1997; Werren and
142 O'Neill, 1997; Zchori-Fein et al., 2001; Zchori-Fein and Perlman, 2004; Zug and Hammerstein,
143 2012]. Some of these are obligate mutualists such as *Buchnera* in aphids [Douglas, 1998; Koga
144 et al., 2003], but many others are reproductive parasites. The most prevalent of these host
145 manipulators are the alpha-proteobacteria *Wolbachia pipientis* and *Rickettsia sp.*, the
146 bacteroidetes *Cardinium hertigii*, the gamma-proteobacterium *Arsenophonus* and the
147 mollicutes *Spiroplasma poulsonii* and *S. ixodetis*, which belong to very distantly related
148 bacterial clades [Duron et al., 2008]. Four broad categories of host reproduction manipulation
149 are distinguished: induction of cytoplasmic incompatibility between egg and sperm,
150 thelytokous parthenogenetic reproduction, killing of male offspring and feminization of
151 genotypic males [Hurst et al., 2002; Werren et al., 2008; Kraaijeveld et al., 2011]. The
152 molecular genetic details of the mechanisms by which these endosymbionts exert the effects
153 on their hosts are not yet well known. Given the diversity of effects and the variety of
154 microorganisms involved, different questions arise: is this true convergence or are horizontal
155 gene transfers between symbionts involved? If this is convergence among symbionts, is it only
156 at the phenotypic level or also at the mechanistic level? How can we explain the seemingly
157 easy evolution of these manipulations? Do different types of manipulation share common

158 mechanisms? Answering these questions requires a better understanding of the molecular
159 mechanisms at play, which in turn will pave the way to better understand the basic processes
160 of sex determination and their evolution. Before getting into these questions we briefly
161 present the different types of reproductive manipulations. The common theme is that host sex
162 determination is somehow manipulated by the endosymbionts to increase their own
163 transmission, which is by vertical transmission through females. Recent evidence suggests that
164 some of these manipulative actions may be attained by directly interfering with host sex-
165 determination genes [Sugimoto and Ishikawa, 2012; Beukeboom, 2012].

166

167 *Cytoplasmic incompatibility*

168 Cytoplasmic incompatibility (CI) is considered as the most widespread endosymbiont
169 manipulation among arthropods [Werren et al., 2008; Kageyama et al., 2012]. It has been
170 found in Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Orthoptera, Isopoda,
171 Trombidiformes, Mesostigmata [Tram and Sullivan, 2002; Werren et al., 2008; Kageyama et al.,
172 2012] (table 1). Despite this broad phylogenetic distribution, CI induction has thus far only
173 been attributed to *Wolbachia* and *Cardinium*. CI is a form of post-zygotic reproductive isolation
174 occurring in crosses between infected males and uninfected females, or when mates harbor
175 different strains of the symbiont [O'Neill et al., 1992; Turelli and Hoffmann, 1995; Werren,
176 1997]. In diploid species, incompatible crosses produce severe cell cycle defects in the male
177 derived pronucleus, resulting in abnormal chromosome condensation at metaphase and
178 aberrant segregation during anaphase of the first mitotic division, which leads to early
179 embryonic mortality [Serbus et al. 2008]. In haplodiploids, CI crosses lead to male-biased
180 offspring sex ratios because elimination of the paternal chromosome set restores haploidy and
181 results in male development [Breeuwer and Werren, 1990, 1993; Breeuwer, 1997;
182 Raychoudhury and Werren, 2012]. However, in some species haploid embryos may also die in
183 an early stage depending on the host species, genotype or the symbiont complement [Vavre et
184 al., 2000, 2001; Perrot-Minnot et al., 2002; Hunter et al., 2003; Mouton et al., 2005], due to
185 the incomplete elimination of paternal chromosomes resulting in aneuploidy, and thus
186 unviable embryos [Tram et al., 2006]. The exact mode of action is not fully understood, but the

187 current model is based on a chromosome marking effect during male gametogenesis that is
188 rescued in the egg if endosymbionts (inherited from the mother via the egg cytoplasm) of
189 similar type are present [Werren et al., 2008]. CI thus results from a delayed paternal effect as
190 *Wolbachia* or *Cardinium* are not present in the sperm. The sequencing of a CI-inducing
191 *Cardinium* genome was expected to provide insights into the mechanisms of CI, but the recent
192 publication of this genome did not bring more information. Interestingly though, it suggests
193 that CI has an evolutionary independent origin in *Wolbachia* and *Cardinium*, since no recent
194 horizontal gene transfer between these two symbionts has been detected [Penz et al., 2012].
195 CI-*Wolbachia* can readily spread in populations, because infected females have an advantage
196 over uninfected females in that they are compatible with uninfected and infected males
197 (Werren 1997).

198

199 *Thelytokous parthenogenesis*

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

216 chromosome complement of the zygote from haploidy to diploidy. Curing of hosts from their
217 endosymbionts with antibiotics typically results in the production of haploid eggs that develop
218 into males.

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

231 PI is the ultimate strategy for a maternally-transmitted symbiont: as fertilization is super-
232 fluous, fixation of the symbiont within populations or entire species is possible. Curing of hosts
233 from their endosymbionts with antibiotics typically results in male production [e.g. Zchori-Fein
234 et al., 2001; Kremer et al., 2009]. However, restoration of sexual lines has yet proved impossi-
235 ble in species in which the endosymbiont is fixed. Sexual traits have decayed either both in
236 males and females, or males partially retain functionality. Two alternative explanations have
237 been proposed. The neutral mutation hypothesis states that if traits involved in sexual repro-
238 duction are neutral under asexuality, relaxed selection might take place and allow mutations
239 to accumulate, for instance, in male sexual traits such as courtship behavior and fertility. The
240 selective hypothesis considers that sexual traits decay can be selected for in females. First, if
241 sexual traits are costly and no longer provide fitness benefits, they are expected to be strongly
242 negatively selected. This applies stronger to female than male sexual traits, like pheromone
243 production, spermatheca functionality, and egg fertilization, because the males are absent
244 under asexuality [Fong et al. 1995; Schwander et al. 2013]. Second, when *Wolbachia* infection

245 remains polymorphic through inefficient transmission of the symbiont, nucleo-cytoplasmic
246 conflict over sex-ratio may select nuclear alleles for higher male production, referred to as
247 “virginity mutants”, which can be achieved through losing the ability to use sperm, or losing
248 the ability to mate [Stouthamer et al., 2010]. Whatever the mechanism at play, PI-symbionts
249 are associated with loss of traits involved in the normal process of sexual reproduction, and
250 this process can be either neutral or actively selected for, which opens up the possibility that
251 endosymbionts take over the role of genes involved in sex determination and sexual differen-
252 tiation.

253

254 *Male killing*

255 Male killing (MK) is induced by a large diversity of endosymbiont taxa and found in a
256 variety of arthropod host orders (table 1). *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Arsenophonus*,
257 *Flavobacteria*, as well as microsporidia have all been reported to cause male killing [reviewed
258 in Hurst and Jiggins, 2000; Kageyama et al., 2012]. Male-killing occurs if sons of infected
259 mothers are killed by the endosymbiont during development [Bonte et al., 2008; Werren et al.,
260 2008]. Endosymbiont-induced male lethality has been reported from six different arthropod
261 orders, i.e. Coleoptera, Diptera, Pseudoscorpiones, Hemiptera, Lepidoptera and Hymenoptera
262 [Werren et al., 2008; Kageyama et al., 2012] (table 1). The MK phenotype is variable and can
263 be divided into two broad categories according to the timing of action: early male killing at
264 embryonic stages and late male killing at late larval or early pupal stages [Hurst, 1991;
265 Kageyama et al., 2007]. Of interest, male-killing is found in species with either male or female
266 heterogamety, as well as haplodiploidy, which suggests, together with developmental timing
267 variation, that male-killing is the outcome of different molecular mechanisms (table 1; fig. 1).
268 Early male-killing is typically encountered in species where intra-brood competition is high;
269 killing brothers allows sisters to have more resources for survival. Late male-killing is
270 associated with parasites having both vertical and horizontal transmission. The
271 microorganisms gain the maximal benefit from it, because male hosts which do not contribute
272 to vertical transmission are killed at the late larval stage when the number of infected cells is
273 maximal allowing for the maximal horizontal transmission [Hurst, 1991; Kageyama et al., 2007;

274 Nakanishi et al., 2008]. Importantly, the presence of male-killing selfish elements leads to
275 selection for host resistance. This is notably what occurred in the butterfly *Hypolimnas bolina*
276 where Asian populations harbor a dominant resistant allele to the male-killing phenotype,
277 although the mechanistic details are not yet known [Hornett et al., 2008]. Interestingly, the
278 rapid spread of resistance has been monitored in natural populations of the South Pacific,
279 highlighting both the dynamic nature of these interactions and the intensity of the selective
280 pressures generated by reproductive manipulators [Charlat et al., 2007].

281

282 *Feminization*

283 Conversion of genotypic males into phenotypic and functional females is known as
284 feminization (FM) [Bouchon et al., 1998; Kageyama et al., 1998]. Endosymbiont-induced
285 feminization has been reported from seven arthropod orders: Lepidoptera, Hemiptera,
286 Hymenoptera, Thrombidiformes, Isopoda, Ephemeroptera and Amphipoda [reviewed in
287 Werren et al., 2008; Kageyama et al., 2012]. Feminization is associated with different sex-
288 determination mechanisms in these groups, such as male or female heterogamety,
289 haplodiploidy, and some unknown mechanisms for crustacean species (table 1). Feminization
290 seems to be more frequent in crustaceans than in insects, which could be due to the easiness
291 to manipulate sexual phenotypes in the former. Indeed, simple manipulation of hormonal
292 levels in crustaceans leads to sex reversion. In the well-studied woodlouse *Armadillidium*
293 *vulgare* (Isopoda), *Wolbachia* feminizes ZZ males by interfering with the
294 production/perception of the androgenic hormone from the male developmental gland during
295 sexual differentiation [Bouchon et al., 2008; Cordaux et al., 2011]. This resembles the shrimp
296 *Gammarus duebeni*, in which microsporidian parasites, such as *Octosporea effeminans* and
297 *Nosema granulosis*, change males into functional females [Bulnheim and Vávra, 1968; Terry et
298 al., 1998; Rodgers-Gray et al., 2004]. Feminization has also been found in insects where
299 different mechanisms could be at play, such as disrupting methylation patterns and genetic
300 imprinting in the male-heterogametic leafhopper *Zyginidia pullula* [Negri et al., 2006, 2009], or
301 altering splicing of *doublesex* in the female-heterogametic butterfly *Eurema mandarina* [Narita
302 et al., 2007]. Feminization also occurs in haplodiploid species. Giorgini et al. [2009] found that

303 in *Encarsia hispida*, curing from *Cardinium* does not lead to haploid but diploid males,
304 suggesting that the endosymbionts are not responsible for genome duplication
305 (parthenogenesis) but rather cause feminization of diploid males. Moreover, in the *Cardinium*
306 infected mite *Brevipalpis phoenicis*, consisting exclusively of haploid females, Weeks et al.
307 [2001] reported that curing of the bacterium changes haploid daughters into haploid sons.

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

318

319

320 **Mechanisms of reproductive and sex determination manipulations**

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

330 how mechanisms of reproductive manipulation may be informative for the molecular bases of
331 host sex determination.

332

333 *Interference with primary signals*

334 Interference with primary sex determination signals concerns notably manipulation of
335 chromosomal behavior. This is clearly established for CI where paternal effects lead to ploidy
336 changes in the early fertilized egg. CI-endosymbionts in diploid arthropod species obviously do
337 not interfere with host sex determination because they cause lethality through haploidization
338 of eggs [Serbus et al., 2008]. However, in haplodiploids, conversion of diploid female eggs into
339 haploid male eggs occurs by changing the zygotic chromosomal constitution that act as
340 primary signal for sex determination. This is very similar to PI-endosymbionts that also act
341 early during sex determination as they alter the number of chromosomal complements at the
342 end of the first or beginning of the second mitotic division. As transcription is probably limited
343 at that time, PI is certainly a parental effect, but contrary to CI, it is limited to a maternal effect.
344 It is still unknown how endosymbionts precisely alter the molecular regulation of mitosis to
345 induce diploidization of the host eggs. Why parthenogenesis inducing microbes have not been
346 found in diploid species remains another mystery. One explanation is that PI evolves more
347 easily in haplodiploids because of the pre-existing cellular machinery of full development from
348 unfertilized haploid eggs. The interaction between mechanisms of sex determination and PI
349 endosymbionts are particularly complex and further elaborated in Box 1. The early MK type
350 can also act on the zygotic chromosome constitution that serves as the primary signal in the
351 host sex-determination pathway. In the wasp *Nasonia vitripennis*, *Arsenophonus nasoniae* kills
352 male offspring by blocking maternal centrosome formation during oogenesis [Ferree et al.,
353 2008]. In *Drosophila bifasciata*, infected male embryos show severe defects of chromatin
354 remodeling and spindle organization, a phenotype strikingly similar to the phenotype
355 observed in CI [Riparbelli et al., 2012].

356 Early acting endosymbionts that alter the chromosomal constitution, a feature of PI, CI and
357 early MK, suggests similar target host genes that have a relatively broad function. This would
358 explain why the manipulations occur in such a diversity of host taxa regardless of their sex-

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

380

381 *Direct interference with doublesex*

382 Late acting endosymbionts are associated with sexual differentiation, and must recognize
383 maleness resulting from male specifically expressed genes during development. It is now
384 evident that endosymbionts can directly interfere with the expression of sex determination
385 genes. For example, male killing in the moth *Ostrinia scapularis*, is accomplished by altering
386 the splicing of *doublesex* [Sugimoto and Ishikawa, 2012]. Altered splicing is also found in the
387 butterfly *Eurema mandarina*, in which *Wolbachia*-infected genetic males (ZZ) are

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

404

405 *Interference during sexual differentiation*

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

417 tea tortrix, *Homona magnanima*, in which male death occurs in the larval or pupal stage
418 [Nakanishi et al. 2008].

419 Hormonal signaling pathways are frequently involved in the regulation of symbiotic
420 interactions. In parasitic interactions such as host-parasitoid relationships, they play a central
421 role in synchronizing host and parasite cycles, and manipulation of hormonal signaling by each
422 party has been found [Sagi and Khalaila, 2001; Negri, 2011; Jahnke et al., 2013]. Hormonal
423 signaling as part of sexual differentiation can also be manipulated by endosymbionts. This is
424 apparent in crustaceans where the establishment of the sexes is a hormonal process. Notably,
425 injection of *Wolbachia* in young males of *A. vulgare* induces the hypertrophy of the androgenic
426 gland and the feminization of tissues [Rigaud and Juchault, 1995]. This result indicates that
427 *Wolbachia* may interfere with the androgenic hormone receptors and either antagonize the
428 fixation of the androgenic hormone on these receptors, or decrease their production. The
429 androgenic hormone is related to insulin and/or insulin-like growth factors, which is
430 interesting for two reasons. First, *Wolbachia* has been shown to interact with the insulin
431 pathway in *Drosophila* [Ikeya et al., 2009]. Even though this pathway is not directly involved in
432 sex determination, insulin-like peptides regulate ecdysteroid synthesis, and recent results
433 indicate that 20hydroxyecdysone could play the role of a sex hormone in insects (Negri et al.,
434 2010; Negri and Pellicchia, 2012). Hormonal manipulation seems mostly associated with
435 feminization, but male-killing may also make use of hormonal signals that are different
436 between the sexes. It should however be noted that sex determination in insects is generally
437 considered as a cellular genetic process, and that the importance of hormonal signaling is still
438 under debate [Steinmann-Zwicky et al., 1989; Schütt and Nöthiger, 2000; Negri and Pellicchia,
439 2012]. This is informative for the transition between MK and FM. The phenotype transition
440 from MK to FM is observed in the moth *Ostrinia scapularis*. Antibiotic treatment induced
441 intersex individuals suggesting that MK-inducing *Wolbachia* were also responsible for
442 feminization [Kageyama and Traut, 2004; Sakamoto et al., 2008; Sugimoto and Ishikawa, 2012].
443 In addition, transition from PI to FM occurred in the parasitoid wasp *Trichogramma kaykai*. In *T.*
444 *kaykai* with PI phenotype, diploid intersex individuals were produced under high temperature,
445 suggesting that PI *Wolbachia* are also responsible for feminization that is *Wolbachia* density

446 dependent [Tulgetske and Stouthamer, 2012] (fig. 2). A small proportion of diploid males is
447 also regularly detected in the parasitoid wasp *Asobara japonica*, which suggests that PI-
448 *Wolbachia* are required for feminization and that this effect is dependent on *Wolbachia*
449 density [W.J. Ma, unpublished data].

450

451 **Box 1 Interaction between PI and FM endosymbionts, and haplodiploid host sex**
452 **determination**

453 The interaction between endosymbionts and haplodiploid host sex determination is
454 complex, because the mechanisms by which diploidization of the egg takes place also affects
455 the outcome. In some cases it dictates whether particular endosymbionts can establish (fig. 3).
456 Several hymenopteran groups have complementary sex determination (CSD) in which sex is
457 determined by the allelic composition of the sex locus: heterozygotes develop into females,
458 hemizygotes and homozygotes into males [Whiting, 1933; Cook, 1993a; Beye et al., 2003]. CSD
459 and PI-inducing endosymbionts that cause gamete duplication are incompatible [Cook, 1993b;
460 van Wilgenburg et al., 2006], because this form of diploidization results in complete
461 homozygosity in most documented species so far [Stouthamer and Kazmer, 1994;
462 Pannebakker et al., 2004; Gottlieb et al., 2002]. The reason is that under CSD diploid
463 homozygotes develop into males whereas female development is required for PI
464 endosymbionts to invade a host. There is indeed a phylogenetic association between absence
465 of CSD and presence of PI endosymbionts [Heimpel and de Boer, 2008]. Interestingly, some
466 CSD species do reproduce parthenogenetically, such as *Venturia canescens*, but in such species
467 the diploidization mechanism is different (e.g. central or terminal fusion) and apparently
468 retains sex locus heterozygosity [Suomalainen et al., 1987; Beukeboom and Pijnacker, 2000;
469 Mateo-Leach et al., 2009]. Functionally apomictic cloning mechanism is also the case for the
470 haploid mite *Bryobia praetiosa* and the parasitoid wasp *Neochrysocharis formosa* [Weeks and
471 Breeuwer, 2001; Adachi-Hagimori et al., 2008].

472 The other known genetic mechanism of sex determination in Hymenoptera is maternal
473 effect genomic imprinting sex determination (MEGISD). Under MEGISD female development

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

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

501

502

503 **Conclusion and future directions**

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

518 With the development of Next-Generation DNA sequencing techniques, it is getting easier
519 to acquire genomic information on non-model organisms, which makes the unraveling of the
520 genetic basis of endosymbiont manipulation very promising and exciting. A first question to
521 answer is whether the diversity of effects and the variation of microorganisms involved,
522 reflects true convergence or merely horizontal gene transfer between symbionts. Future
523 studies should compare different endosymbiont genomes for gene composition, as well as
524 compare gene products that might affect developmental pathways of their host [e.g. Moreno
525 et al. 2011]. For instance, the comparison of genomes between *Wolbachia* and *Cardinium*
526 suggests that CI has an evolutionary independent origin in these two symbionts and reveals no
527 evidence for recent horizontal gene transfer [Penz et al., 2012]. Comparison of transcriptomes
528 and proteomes of infected and uninfected hosts may also be rewarding [e.g. McNulty et al.
529 2012]. Moreover, the integration of knowledge about evolutionary dynamics and genomic

530 data should make it possible to identify genomic signatures that can lead to the identification
531 of genes involved in host reproduction and sex determination manipulation. Attention should
532 be paid to host sex determination genes such as *transformer* and *doublesex*, candidate targets
533 for disruption by endosymbionts, whose regulation may be altered in several ways, including
534 their sex-specific splicing or imprinting. In addition, cell cycle genes involved in meiosis and
535 mitosis, particularly those related to histone regulation or genes coding for Argonaute proteins
536 are good candidates (Kraaijeveld and Bast, 2012).

537 There is growing evidence for epigenetic control of developmental processes in insects [Lyko
538 & Maleszka 2011], as well as in host-parasite interactions [Gómez-Díaz et al., 2012]. Given the
539 multiple evolution of reproductive manipulations, it is tempting to propose that these
540 phenotypes may actually be mechanistically very close to other physiological mechanisms
541 involved in host-parasite interactions that could represent pre-adaptations to reproductive
542 manipulations [Vavre et al., 2003]. As reproductive manipulations often involve parental
543 effects, epigenetic manipulation by endosymbionts clearly requires attention. An obvious
544 candidate is chromatin remodeling which can lead to alteration of chromosomal behavior, as
545 well as to variation in gene expression or splicing processes. It is thus possible that many of
546 the symbiont phenotypes rely on epigenetic mechanisms, particularly those related to histone
547 regulation. Moreover, paternal effects of CI, maternal effects of PI, and mechanisms of early
548 MK, may all involve some form of genomic imprinting [Werren 2011; Negri and Pellecchia,
549 2012; Rabeling and Kronauer, 2013]. The currently strongest evidence for a role of epigenetics
550 was found by Negri et al. [2009], who showed that *Wolbachia* interferes with host sexual
551 differentiation in the leafhopper *Zyginidia pullula* by disrupting methylation patterns and
552 genetic imprinting. In *Drosophila* species, *Wolbachia* prophage DNA adenine
553 methyltransferase genes might be involved in the modification or rescue process of CI
554 [Saridaki et al., 2011]. These studies are first indications for a role epigenetics in host
555 manipulation, but we are only at the beginning of elucidating the precise molecular and
556 biochemical pathways involved. Technological developments now allow for easier
557 characterization of epigenetic marks, and transcriptome and proteome comparison of infected
558 and uninfected individuals in various systems may be a promising way forward. Without doubt,

559 more mechanistic studies of host reproduction manipulation are going to reveal novel and
560 intriguing insights into the co-evolution between host and endosymbiont reproduction.

561

562

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896 **Titles and legends to figures**

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

909

910 **Fig. 2.** Transitions between the four different manipulative phenotypes of *Wolbachia*. FM:
911 feminization, EMK: early male killing, LMK: late male killing, CI: cytoplasmic incompatibility
912 and PI: thelytokous parthenogenesis induction. The reported species (and orders) are
913 indicated at each arrow, as well as their mode of sex determination.

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915 **Fig. 3.** PI-inducing endosymbionts and haplodiploid host sex determination. CSD:
916 complementary sex determination, MEGISD: maternal effect genomic imprinting sex
917 determination. Red arrows: incompatible combinations. Green arrows: compatible
918 combinations. CSD is only compatible with PI if diploidization is other than by gamete
919 duplication (e.g. premeiotic doubling, central fusion or terminal fusion). MEGISD species can
920 only have PI if the maternal imprint, that prevents female development, is not copied during
921 gamete duplication, or the endosymbionts remove it before or after diploidization. The mode
922 of sex determination and diploidization are mutually informative on each other: in *Lepidoptera*
923 *clavipes* and *Trichogramma kaykai*, PI occurs by gamete duplication and CSD is excluded as sex

924 determination [Schilthuizen et al., 1998; Pannebakker et al., 2004; Tulgetske, 2010]; in
925 *Asobara japonica* CSD is absent and diploidization could be by gamete duplication [Kremer et
926 al., 2009; Ma et al., 2013]; and in *Encarsia hispida* feminization of diploid males can occur
927 under MEGISD if *Cardinium* removes the imprint [Giorgini et al. 2009].

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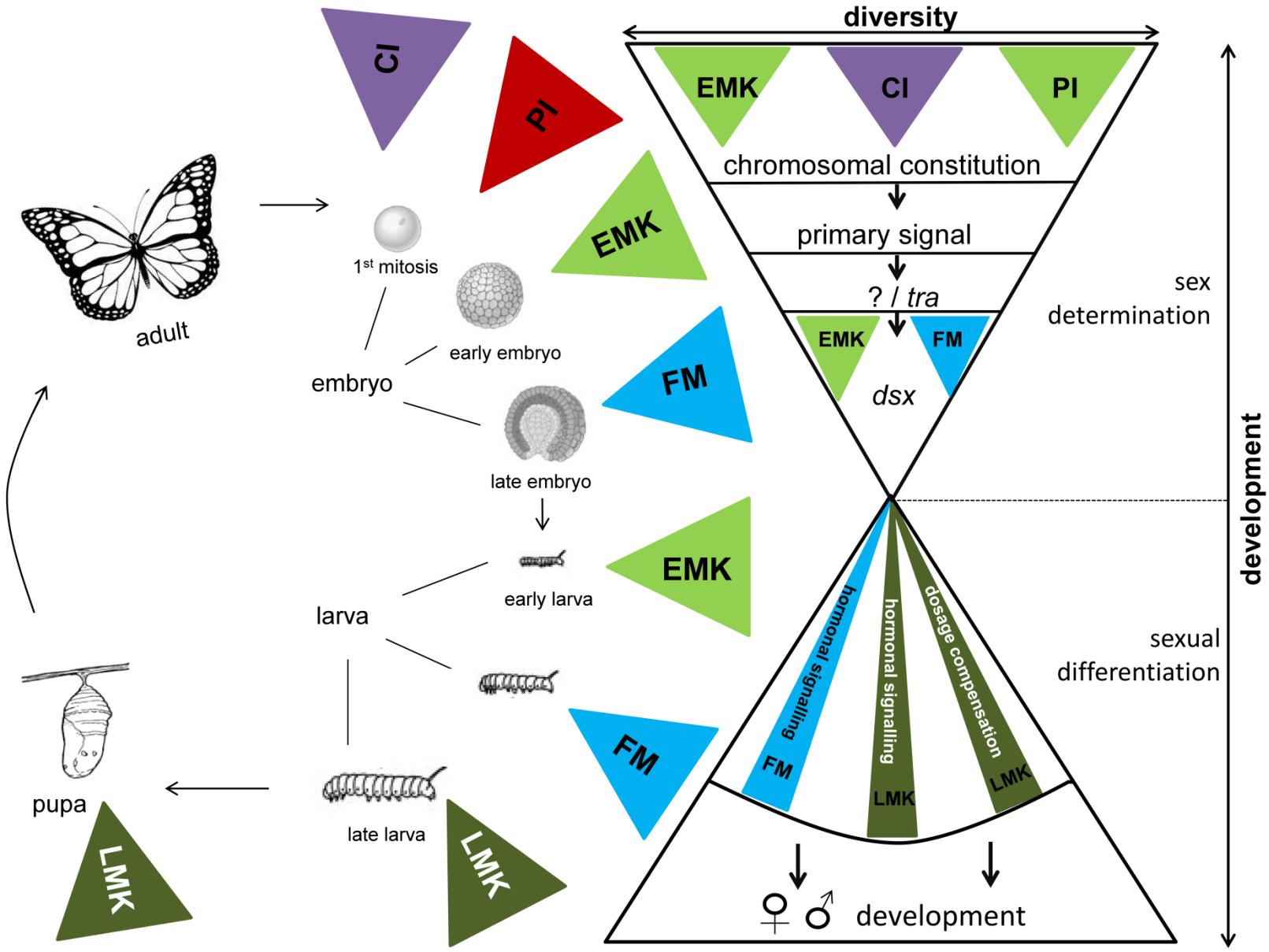
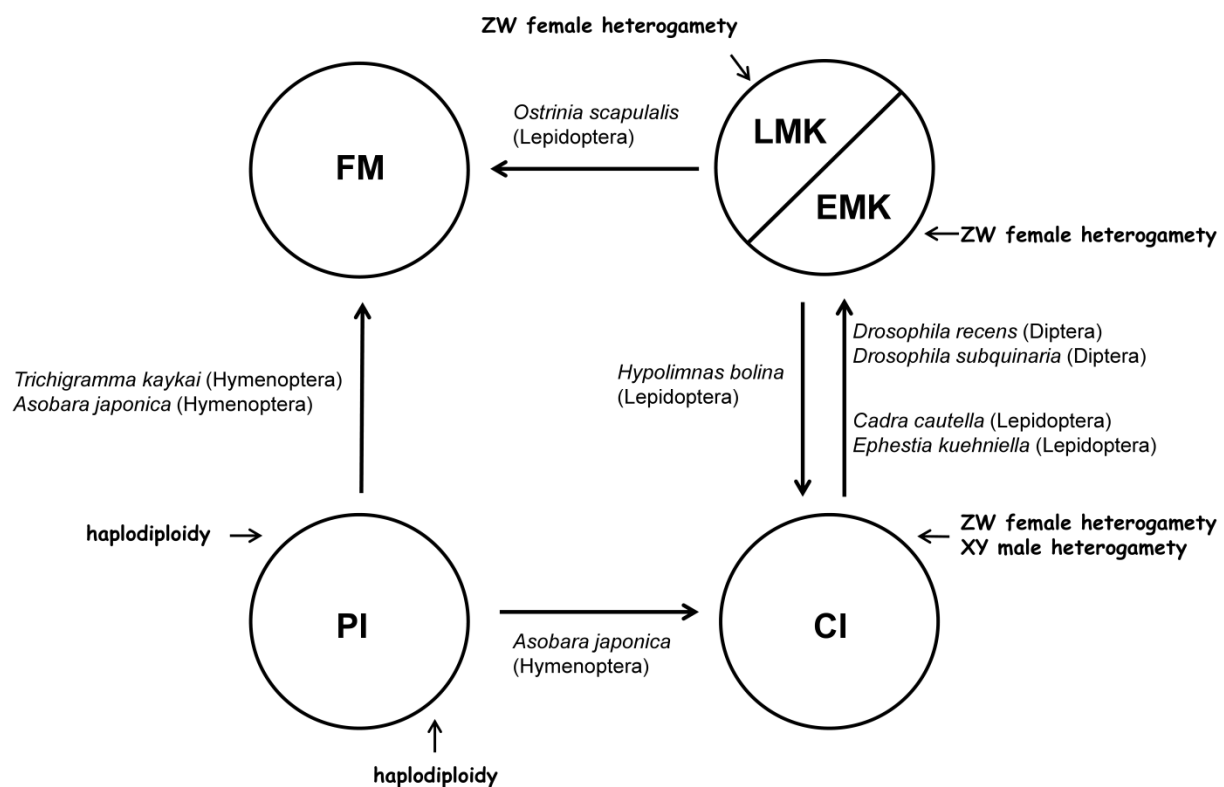
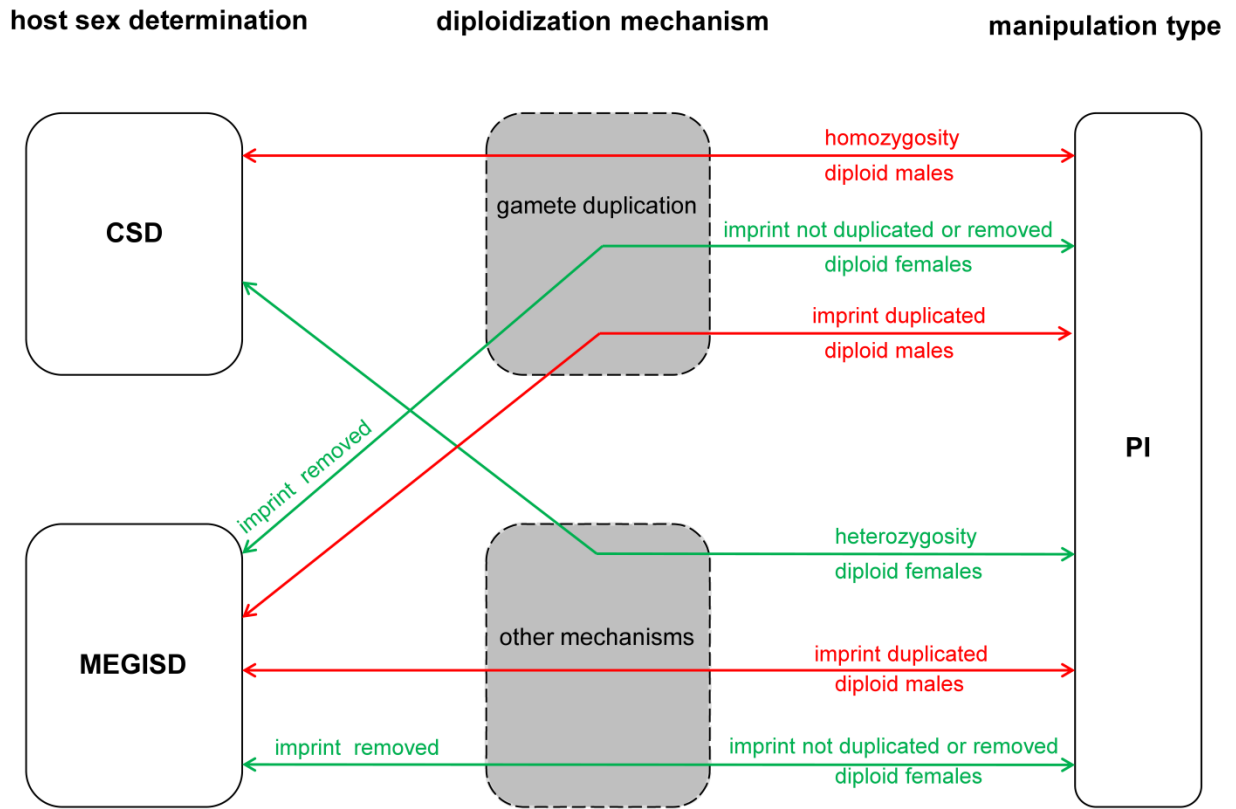


Fig. 1.



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

Manipulation phenotype	Endosymbiont	Arthropod host order	Host sex determination (number of species reported)
Cytoplasmic incompatibility (CI)	<i>Wolbachia</i> <i>Cardinium</i>	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 (PI)	<i>Wolbachia</i> <i>Cardinium</i> <i>Rickettsia</i>
Thysanoptera	haplodiploidy (1)		
Trombidiformes	haplodiploidy (2)		
Male killing (MK)	<i>Wolbachia</i> <i>Spiroplasma</i> <i>Rickettsia</i> <i>Arsenophonus</i> <i>Flavobacteria</i> Microsporidia parasites unknown virus	Coleoptera	XY male heterogamety (4) ZW female heterogamety (4) Unknown (3)
		Diptera	XY male heterogamety (14)
		Pseudoscorpiones	XO male heterogamety (1)
		Hemiptera	XO male heterogamety (1)
		Lepidoptera	ZW or ZO female heterogamety (13)
		Hymenoptera	Haplodiploidy (1)
		Feminization (FM)	<i>Wolbachia</i> <i>Cardinium</i> Microsporidia parasites <i>Gasteromermis</i> f factor (unknown)
Hemiptera	XO male heterogamety (1)		
Hymenoptera	haplodiploidy (2)		
Trombidiformes	haplodiploidy (3)		
Isopoda	ZW female heterogamety (2)		
	XY male heterogamety (1)		
	unknown (2)		
Ephemeroptera	unknown (1)		
Amphipoda	unknown (4)		

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