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Variation in sex determination mechanisms may constrain parthenogenesis-induction by endosymbionts in haplodiploid systems

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Endosymbionts are maternally transmitted, and therefore benefit from maximizing female offspring numbers. Parthenogenesis-induction (PI) is the most effective type of manipulation for transmission, but has solely been detected in haplodiploid species, whereas cytoplasmic incompatibility (CI) is detected frequently across the arthropod phylum, including haplodiploids. This puzzling observation led us to hypothesize that the molecular sex-determination mechanism of the haplodiploid host may be a constraining factor in the ability of endosymbionts to induce parthenogenesis. Recent insights indicate that PI-endosymbionts may be able to directly manipulate sex-determination genes to induce the necessary steps required for PI in haplodiploids. However, sex-determination cascades vary extensively, so PI-induction would require a specialized and host-dependent tool set. Contrastingly, CI-related genes target conserved cell-cycle mechanisms, are located on mobile elements, and spread easily. Finally, endosymbiont-manipulations may have a strong impact on the effectiveness of haplodiploid biocontrol agents, but can also be used to enhance their efficacy.

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Introduction

Within the arthropod world, a majority of species are infected with one or more endosymbionts of which *Wolbachia* is the most prevalent [1]. These endosymbionts live inside the cells of their host, and as such can only be transmitted to the next generation through the cytoplasm of the egg, in a process called vertical transmission. The spermatids cannot contain endosymbiont cells, and hence the males do not contribute to their transmission. Therefore, endosymbionts manipulate the reproduction of their host by increasing the number of female offspring and maximize their transmission in the population. Endosymbionts can also be horizontally transmitted between hosts through consumption or superparasitism (such endosymbiont host shifts are extensively reviewed in [2]).

Endosymbionts have four described mechanisms by which they manipulate the reproduction of their host to maximize their transmission: cytoplasmic incompatibility (CI), feminization, male-killing, and thelytokous parthenogenesis-induction (PI). CI causes embryonic lethality in diplodiploid crosses between infected males and uninfected females, and causes male-biased offspring in haplodiploids, reducing the fitness of uninfected females. Feminization causes genetic males to develop into fully functional females, but a low number of males is still required in the population for sexual reproduction. Male-killing is the selective killing of males resulting in a female-biased offspring sex ratio. PI causes infected females to asexually produce daughters, meaning that all potential offspring develops as actively reproducing females, and there is no fitness loss due to offspring death (reviewed in [3]). Based on the available data, CI is most prevalent in arthropods, while feminization and male-killing seem restricted to certain species. PI is thus far only confirmed in haplodiploid species (but see [4] for examples of diplodiploid species with suspected endosymbiont-induced parthenogenesis). In addition, haplodiploidy may constrain endosymbionts to only PI or CI-induction. Because theoretically PI maximizes endosymbiont transmission to the next generation, we would expect that PI would be the most prevalent type of reproductive manipulation in haplodiploids, but thus far we find no evidence for this. Many different selection forces act on the conflict between the

endosymbiont and its host for reproduction, for which the molecular mechanism of the host sex determination system forms the foundation [5]. We hypothesize that the sex-determination mechanism of the host may be a constraining factor in the ability of endosymbionts to induce parthenogenesis, the most effective form of reproductive manipulation in terms of transmission potential. As recently several studies on the sex determination mechanisms in haplodiploid parasitoid wasps (Hymenoptera) became available, and PI is thus far only confirmed in haplodiploids, we focus our review on haplodiploid wasps.

Endosymbionts induce parthenogenesis using a one-step or two-step mechanism

Endosymbiont-mediated parthenogenesis leads to thelytokous development of an unfertilized, haploid egg into a diploid female. In the parasitoid *Asobara japonica* infected with PI-*Wolbachia*, it was shown using an increasing concentration of antibiotics that PI is a two-step mechanism [4]. Higher antibiotics concentrations result in a lower amount (titer) of *Wolbachia*. Mothers with low *Wolbachia* titers in their ovaries produce haploid males, while mothers with intermediate *Wolbachia* titers were shown to produce diploid males. This shows that diploidization is induced through an unknown endomitosis mechanism for which relatively low titers are sufficient. When high *Wolbachia* titers are present, it induces feminization of the diploid zygote as well [6]. Similarly, in *Trichogramma kaykai* spontaneous diploid males and intersexes were observed in *Wolbachia*-infected laboratory lines, also suggesting a two-step mechanism in this species (GM Tugetske, PhD thesis, University of California, Riverside, 2010). Intermediate *Wolbachia* titers in *Trichogramma pretiosum* seem to result in an increased percentage of intersexes, suggesting that *Wolbachia* regulates the feminization-step separately from the diploidization-step here also [7].

A feminization-step without diploidization is detected in the parasitoid *Encarsia hispida* [8], and in the false spider mite *Brevipalpus phoenicis* [9]. In *E. hispida*, antibiotics removal of its *Cardinium* endosymbiont results in diploid male offspring, suggesting that *Cardinium* only induces the feminization-step. In *B. phoenicis*, removal of its *Cardinium* infection results in the production of haploid males. However, the females are also haploid, and the endosymbiont again only induces the feminization-step [9]. In *E. hispida*, it is likely that *Cardinium* once induced parthenogenesis in two steps: diploidization in the first step and feminization in the second step. Perhaps the gene(s) required for diploidization were horizontally transferred into an ancestral *E. hispida* genome, and *E. hispida* females are now themselves capable of gamete diploidization. For *B. phoenicis*, the origin of its haplo-haploidy is unclear. However, in both cases, the feminization-step in these groups is part of the parthenogenesis induction as the females produce females without the need for males.

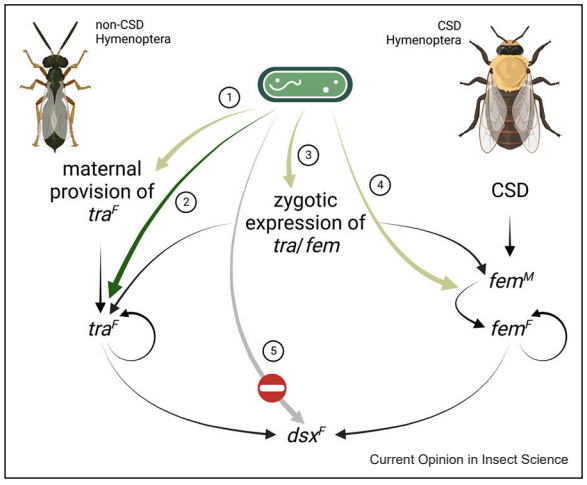
In contrast, in the *Wolbachia*-infected species *Leptopilina clavipes* and *Muscidifurax uniraptor*, similar experiments with antibiotics treatments did not produce diploids males [10] (Y Wang et al., bioRxiv doi: 10.1101/2022.10.27.514028). At the *Wolbachia* titer switch point, individual females would produce diploid females and/or haploid males, but never diploid males. This suggests that either PI happens in one step, with the diploidization-step automatically leading to female development, or that the diploidization-step and the feminization-step happens with the same endosymbiont titer, and this cannot be separated [10]. For *M. uniraptor*, it was shown that PI must be a one-step mechanism with the diploidization-step automatically leading to female development, as triploid females of its noninfected sister species, *Muscidifurax raptorellus*, produced uniparental diploid offspring that developed into females (Y Wang et al., bioRxiv doi: 10.1101/2022.10.27.514028). This suggests that being diploid is sufficient to initiate female development and that the endosymbiont only needs to induce diploidization in *Muscidifurax* wasps. For *L. clavipes*, it is still unclear what the mechanism is, but knowledge of its sex determination mechanism could be informative (see below).

Ma and Schwander [4] compiled all up-to-then described cases of endosymbiont-PI, and in most cases verification using antibiotics treatment was done. Yet, researchers often overlook to study the ploidy of the resulting males. For example, in *Encarsia formosa*, researchers have studied endosymbiont titers and their effect on sex determination, however the ploidy of the resulting males was not established [11]. Thus, it remains unclear whether *E. formosa* has a one- or two-step PI mechanism. Future research into endosymbiont-PI should include an antibiotics-gradient experiment combined with ploidy measurements of the resulting males to verify a one- or two-step mechanism of PI. This would give much more insight into the host's sex-determination mechanism, which is essential for gaining a better understanding of the constraints that endosymbionts may encounter when inducing parthenogenesis.

Parthenogenesis-induction often requires endosymbionts to interact with sex determination genes

For the active induction of the feminization-step in PI, endosymbionts need to manipulate one or more genes in the sex determination cascade. All insect cascades are conserved downstream but highly variable upstream [12,13]. In many insects, *transformer* (*tra*) is the central part of the cascade. It is alternatively spliced into

Figure 1



Possible routes by which PI-endosymbionts could induce the feminization-step after diploidization induction. 1) Enforce maternal provision of tra^F; 2) Initiate or maintain the autoregulatory loop of tra^F splicing by for example improving efficiency of tra^F splicing (as shown in *L. clavipes* [20]); 3) Increase zygotic expression of *tra/fem* required for autoregulation of tra^F splicing, but requires endosymbiont to mimic the correct transcription factor, and depends on maternal provision of tra^F; 4) Bypass CSD by initiating fem^F splicing; 5) Enforce splicing of dsx^F, endosymbiont would need to mimic a *dsx* splicing factor *and* be present in all sex-specific tissues and developmental stages to continue enforcing dsx^F splicing. We consider this nonparsimonious. Dark green arrow indicates empirically shown route, light green arrows indicate hypothetical routes for endosymbiont-induced feminization, gray arrow indicates an nonparsimonious route.

female-specific transcripts yielding functional TRA^F protein, or into male-specific transcripts that encode truncated nonfunctional TRA^M protein. In most insects, TRA^F also directs its own splicing into the female-specific mode, thereby creating an auto-regulatory loop which memorizes the female mode once this is initiated. TRA^F also directly regulates female-specific splicing of *doublesex* (*dsx*), while nonfunctional TRA^M in males leads to default splicing of *dsx*. The resulting sex-specific proteins execute the male or female differentiation program in a time and place dependent manner [14,15]. Although *dsx* is highly conserved in insects and would seem to be a central conserved target for endosymbiont manipulation, this would require the endosymbiont to be present in all sex-specific tissues and all developmental stages to continuously control dsx^F splicing (Figure 1).

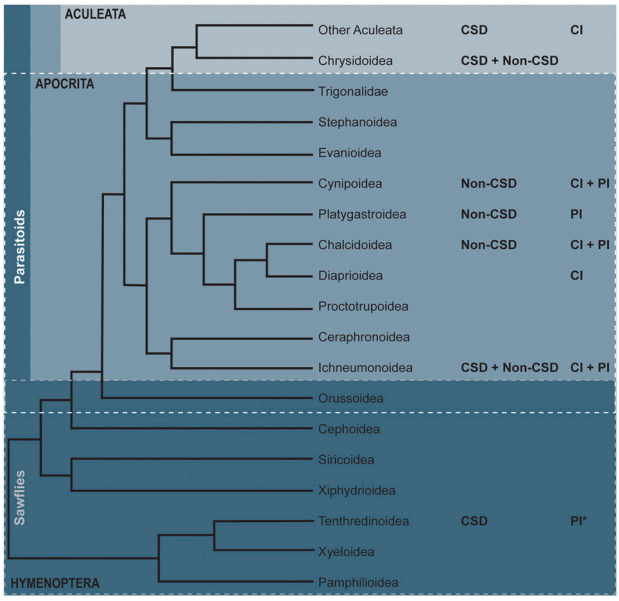
In many haplodiploids, *tra* transcripts (and potentially protein) are maternally provided to the eggs [16]. Maternally provided splice variants range from a predominant female-splice variant [17](Y Wang, PhD thesis, Wageningen University, 2021), to a nonsex-specific splice variant [18], and a male-specific splice variant [19]. Recent work on parasitoid wasps has shown that *Wolbachia* may manipulate sex-specific splicing at this level, as *Leptopilina clavipes* females infected with PI-*Wolbachia* solely contain female-specific *tra* (tra^F) transcripts, in contrast to their noninfected counterparts [20]. This may indicate that PI-*Wolbachia* is capable of driving *tra* splicing to the female variant to initiate feminization by bypassing any initial instructive signal (Figure 1). We suggest on this basis that *L. clavipes* has a two-step mechanism of PI, as was shown for *A. japonica* [6]. However, in *M. uniraptor*, PI-*Wolbachia* does not manipulate the already existing maternal provision of tra^F to the eggs, and indeed here a one-step mechanism has been strongly suggested(Y Wang et al., bioRxiv doi: 10.1101/2022.10.27.514028). Similarly, *Nasonia vitripennis* also provides maternal tra^F to their eggs [17] and has no known PI-endosymbiont. Both systems rely on ample expression of zygotic *tra* expression for female development, with a lack of zygotic *tra* expression leading to male development [17] (Y Wang, PhD thesis, Wageningen University, 2021). Maternal provisioning of tra^F may complicate or even negate the establishment of endosymbiont-PI in these types of systems, unless the endosymbiont can initiate zygotic *tra* expression (Figure 1). As the *tra* transcription factors seems highly variable, endosymbionts mimicking these transcription factors would need an extensive toolset.

Complementary sex determination (CSD) is a mechanism present in a subset of Hymenoptera [21]. In these species, females are heterozygous, and males are hemizygous at one or more CSD loci. Homozygosity at this locus, for example due to inbreeding, leads to diploid male development. All known cases of *Wolbachia*-PI rely on gamete duplication for the diploidization step [4]. As gamete duplication leads to full homozygosity, and homozygosity at the CSD locus leads to diploid male development, *Wolbachia*-PI, and CSD were considered incompatible. However, in honeybees, CSD protein initiates female-specific splicing of *feminizer* (*fem*; *tra* ortholog) and *fem* maternal provision is male-specific [19]. This may allow *Wolbachia* to induce PI by enforcing female-specific splicing of *fem* independent of the allelic state of CSD. Diploidization mechanisms that retain full or partial heterozygosity could be compatible with CSD directly without the need to bypass this initial signal, and these have been observed in other endosymbionts (see [4]). Although theoretically possible, cases of PI-endosymbionts infecting CSD systems have not been observed yet.

Occurrence of cytoplasmic incompatibility and parthenogenesis-induction in parasitoids (and some nonparasitoid Hymenoptera)

In haplodiploids, CI leads to a more male-biased sex ratio instead of full offspring mortality, as unfertilized embryos do survive and develop into haploid males, and

Figure 2



Schematic overview of CI, PI, and CSD presence in Hymenoptera phylogeny. Phylogenetic relationships based on [34]. The Apoidea, Formicoidea, Scolioidea, Pompiloidea, Thynnoidea, Tiphioidea, and Vespoidea are merged in the branch "Other Aculeata" as no mechanisms besides CSD have been detected in this group. Two cases of (possible) CI have been detected in the Aculeata *Acromyrmex insinuator* [35] and *Cardiocondyla obscurior* [36]. Examples of CI cases are described in [28,29,37,38]. Presence/absence of CSD based on [39] and endosymbiont phenotypes on [4]. *Possible presence of PI in Tenthredinoidea is hypothesized from temperature effects on daughter production.

only the fertilized eggs cease to develop. In addition, some fertilized eggs undergo complete haploidization due to CI and develop as haploid males. CI can mechanistically be explained by a toxin-antidote model or a host-modification model [22–24]. Neither requires manipulation of the sex determination pathway, and the underlying mechanism of sex determination is not expected to limit the distribution of CI in Hymenoptera. Indeed, CI has been detected in multiple hymenopteran families (Figure 2), but detection is often limited by experimental and rearing limitations, such as for the sawflies or Aculeata.

CI phenotypes have been linked to genetic elements located in the phage WO (reviewed in [25]). Comparative genomics reveal that the CI factors (*cif*)genes responsible for CI in Diptera, are also conserved in *Wolbachia* infecting hymenopteran hosts [26]. *Cif* gene expression in the wasp *Habrobracon hebetor* was consistent with patterns detected in *D. melanogaster* [27,28]. Other endosymbionts also induce CI in parasitoids, but the CI-inducing extracellular symbiont *Spiroplasma* in *Lariophagus distinguendus* lacks *cif* genes [29]. Similarly, CI-*Cardinium* does not contain *cif genes* and, in *Encarsia partenopea*, is present in different tissues compared to other CI-inducing symbionts [30,31]. The apparent diversity of CI mechanisms and CI-endosymbionts suggests multiple independent cases of CI emergence. In addition, the localization of *cif* genes on phage WO enables wide and rapid transmission between *Wolbachia*. *Cardinium* and *Spiroplasma* may contain other CI-inducing genes with similar mobility.

PI is detected in several branches of the Hymenoptera (Figure 2), but is not very abundant, and some cases of thelytokous reproduction are of nuclear, rather than symbiont, origin. The infrequent presence may indicate that PI does not transmit as rapidly as other reproductive manipulations by endosymbionts. Potentially, PI genetic elements are not located in phage WO, but additionally they may require further alterations to target the ever-changing sex determination cascade of the host. Furthermore, PI-endosymbionts are not detected in the Aculeata and may be incompatible with the dual female developmental pathways leading to queens and workers. Also, there is no direct evidence that switches between PI and CI occur, but limited evidence suggests that PI-*Wolbachia* can also induce CI in the wasp *A. japonica* [32]. Theoretical models have demonstrated that CI-inducing endosymbionts are highly susceptible to invasion by sex ratio distorting mutants [33]. This may indicate that CI is a precursor for PI and that, based on their mitotic effects, CI genetic elements may be linked to the diploidization-step necessary for PI.

Biocontrol application of parthenogenesis-induction or cytoplasmic incompatibility endosymbionts

Parasitoid wasps are the main group of haplodiploids used for biological control, as the females lay eggs in or on another (pest) insect. For efficient biocontrol purposes, having increased fitness and a strong female-biased sex ratio in the population is advantageous. Therefore, knowledge on the presence of CI- or PI-endosymbionts and their mechanisms is not just of fundamental interest. For example, many *Trichogramma* species infected with PI-*Wolbachia* can still reproduce sexually. Infected *T. pretiosum* females produce diploid homozygous daughters when virgin, but produce also heterozygous diploid daughters when mated with a male from an uninfected line. These heterozygous daughters are also infected and have thelytokous reproduction when virgin, creating 'recombinant' introgression lines. This enables fast introgression of optimal biocontrol traits in existing strains, but also fixation of the trait over multiple generations, resulting in predictable biocontrol performance [27,40]. PI-endosymbiont presence can also have a beneficial impact on parasitoid biocontrol effectiveness solely by

increasing the number of females in production [41,42]. However, because it can also result in other fitness costs to the female parasitoid [43,44], the potential advantage to biocontrol programs needs to be considered for each case specifically. Similarly, the presence of CI endosymbionts requires careful consideration when releasing biocontrol agents. A nonmatch between the CI strain of the released parasitoids and that of the local population can result in a reduction in population growth rate, and hence in control efficiency [37,45].

Knowledge of the specific genes used by PI endosymbionts to induce feminization could be applicable for the optimization of biocontrol agent sex ratios for mass rearing and release. If those genes turn out to be generic, they could be applied broadly. Understanding the novel establishment of endosymbionts in a new host could aid in trans-infections as has already been mainly done with CI *Wolbachia* in mosquitoes [46,47]. This knowledge is much needed for PI-endosymbionts to extend PI to noninfected but highly effective biocontrol agents.

Conclusion and outlook

We started with the hypothesis that the variability of molecular sex determination mechanisms poses a constraint for endosymbionts to induce parthenogenesis in their haplodiploid host. In both one- or two-step PI mechanisms, the gamete needs to be diploidized by endosymbiont-modification of meiosis or mitosis (reviewed in [30]), but more research is needed to fully unravel these mechanisms. In CI, histones and other proteins involved in chromatin formation prior to the first mitotic division may be involved. Expression of *cif* genes in modified yeast demonstrates CI effects and indicates that CI components target highly conserved mitotic processes [48]. Therefore, PI and CI phenotypes seem to converge on the host cell cycle and it could be that both mechanisms target the same set of highly conserved genes involved in the metaphase of the cell cycle to induce CI or the diploidization-step.

A spur of research into the *cif* genes underlying CI has tremendously advanced our insight into the widespread nature of CI. The mere fact that in *Wolbachia cif* genes are located on phage WO aid in its transmission to other *Wolbachia* strains, and most likely accounts for its high prevalence [25]. However, not all CI-endosymbionts contain *cif* genes, and more research is needed to fully understand the molecular nature of those CI systems. Genomic data of PI-endosymbionts is lacking compared to their CI counterparts and further investigations would be required to elucidate whether diversity in PI genes underlie the different varieties of PI mechanisms.

For PI, either diploidization alone is sufficient, or a separate feminization-step is required, potentially via direct manipulation of sex-determination genes. A few recent studies now started to shed light on the interplay between PI-endosymbionts and the sex-determination system of the host. *Wolbachia* endosymbionts may manipulate splicing of sex-determination genes, and hypothetical routes for sex-determination manipulation are plentiful. However, each of these likely requires its own specialized tool set, which would need to be host dependent and rapidly evolving. This contrasts CI which only needs to target the highly conserved cell cycle, and additionally, CI genes appear often located on mobile elements allowing for a rapid transmission.

Ultimately, much more research is needed on the molecular basis of haplodiploid sex determination cascades in species with endosymbionts and without endosymbionts before we can truly infer a relationship between the presence of CI- or PI-endosymbionts and the sex determination system of the host. This will also aid in unraveling the diversity of endosymbiont mechanisms, and shed light on their distribution throughout haplodiploid insects.

Data Availability

No data was used for the research described in the article.

Declaration of Competing Interest

None.

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Glossary

Complementary Sex Determination (CSD): System of sex determination in Hymenoptera, where complementation of alleles at a sex locus determines the sex of the offspring. Hemi- or homozygosity at this locus results in haploid or diploid male development, respectively, and heterozygosity at this locus in female development.

Cytoplasmic Incompatibility (CI): Endosymbiotic manipulation of host reproduction where sperm of infected males is incompatible with the egg of uninfected females, resulting in the death of the zygote in the case of diplodiploid organisms, or haploid male development in the case of haplodiploid organisms.

Cif genes: CI factor encoding genes, which are located in phage WO and are responsible for cytoplasmic incompatibility.

Diplodiploidy: Life cycle where both males and females develop from fertilized eggs and are diploid.

Doublesex: Highly conserved transcription factor at the end of the sex determination cascade, that executes the male or female differentiation program in a time and place dependent manner.

Feminization: Endosymbiotic manipulation of host reproduction where genetic males develop into fully functional females.

Haplodiploidy: Life cycle where males are haploid and develop from unfertilized eggs, and females are diploid and develop from fertilized eggs.

Haplohaploidy: Life cycle where both males and females are haploid. Thus far only identified in the false spider mite *Brevipalpus phoenicis (Weeks et al. 2001)*.

Male-killing: Endosymbiotic manipulation of host reproduction where males are selectively killed.

Parthenogenesis Induction (PI): Endosymbiotic manipulation of host reproduction where unfertilized eggs develop into females.

Thelytoky: Form of parthenogenetic reproduction where unfertilized eggs develop into diploid females. Can be induced by endosymbionts or through nuclear factors.

Transformer: Central switch gene of the sex determination cascade in many insects. In females the gene is sex-specifically spliced yielding $TRA^{F,}$ and is required for female-specific *Dsx* splicing. In males the gene is sex-specifically spliced to produce the nonfunctional TRA^{M}. TRA^{F} can also directs its own splicing into the female-specific mode, thereby creating an auto-regulatory loop.