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Wolbachia as an "infectious" extrinsic factor manipulating host signaling pathways

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Wolbachia pipientis is a widespread endosymbiont of filarial nematodes and arthropods. While in worms the symbiosis is obligate, in arthropods *Wolbachia* induces several reproductive manipulations (i.e., cytoplasmic incompatibility, parthenogenesis, feminization of genetic males, and male-killing) in order to increase the number of infected females. These various phenotypic effects may be linked to differences in host physiology, and in particular to endocrine-related processes governing growth, development, and reproduction. Indeed, a number of evidences links *Wolbachia* symbiosis to insulin and ecdysteroid signaling, two multilayered pathways known to work antagonistically, jointly or even independently for the regulation of different molecular networks. At present it is not clear whether *Wolbachia* manipulates one pathway, thus affecting other related metabolic networks, or if it targets both pathways, even interacting at several points in each of them. Interestingly, in view of the interplay between hormone signaling and epigenetic machinery, a direct influence of the "infection" on hormonal signaling involving ecdysteroids might be achievable through the manipulation of the host's epigenetic pathways.

Keywords: *Wolbachia*, insulin, ecdysone, nuclear receptors, epigenetic

INTRODUCTION

The maternally transmitted alfa-Proteobacterium *Wolbachia pipientis* (Rickettsiales) is a widespread endosymbiont of filarial nematodes and arthropods, including crustaceans, mites, spiders, scorpions, and especially insects, where it is estimated to infect up to 66% of the species (Werren et al., 2008).

While in worms *Wolbachia* are obligate symbionts, in arthropods they induce several reproductive manipulations in order to increase the number of infected females. Effects of the infection include cytoplasmic incompatibility, parthenogenesis, feminization of genetic males, and male-killing, as well as influences on host longevity and fecundity (Stouthamer et al., 1999; Werren et al., 2008). Such a host phenotypic variability is generally linked to the high genome plasticity of *Wolbachia*. However, experimental data also suggest a role for the symbiont in modulating the host sexual phenotypes by interaction with the hormonal signaling pathway. In particular, the various phenotypic effects may be due to differences in host physiology, and in particular to endocrine-related processes governing growth, development, and reproductive behavior which display a high variability in insects. In particular, a number of evidence links *Wolbachia* symbiosis to insulin and steroid (i.e., ecdysteroid) signaling. Many studies report antagonistic or cooperative relationships between steroid hormones and insulin. Indeed, an extensive crosstalk between the two hormonal signaling pathways has been demonstrated in regulating metabolism, development, and reproduction. For example, in the fly *Drosophila melanogaster* there is a mutual antagonistic relationship between these metabolic networks for nutrient homeostasis (Colombani et al., 2005). At a molecular level, for example, the expression of the ecdysone receptor (EcR) co-activator DOR (which is misregulated in diabetic mammals) is controlled by insulin signaling via the forkhead transcription factor FOXO (Francis et al., 2010). The two hormonal signaling pathways may also cooperate: in mosquitoes they act in combination for the yolk protein precursor gene expression required for vitellogenesis (Roy et al., 2007). In the larval prothoracic gland, the insulin-signaling pathway modulates ecdysone release and influences both the duration and rate of larval growth (Shingleton, 2005); in adults, insulin-like peptides may trigger steroid synthesis by the follicular cells in insect ovaries (Wu and Brown, 2006). A parallel regulation by ecdysone and insulin has also been demonstrated in the *Drosophila*'s ovary where the hormones modulate the proliferation and self-renewal of germ-line stem cells independently (Ables and Drummond-Barbosa, 2010).

In the following sections, data on the involvement of *Wolbachia* in modulating both host hormonal pathways are discussed. Due to the complexity of such metabolic pathways, at present, it is not clear whether *Wolbachia* operates by attacking one pathway, thus affecting other related metabolic networks, or if the symbiont targets both pathways, even interacting at several points in each of them.

WOLBACHIA AND INSULIN SIGNALING

The insulin/IGF signaling (IIS) pathway plays key roles in growth, metabolism, reproduction, and longevity in different organisms. Recently, a specific interaction between IIS and *Wolbachia* has been demonstrated in *D. melanogaster*, where insulin-like peptide mutants display an extended lifespan if they harbor the symbiont (Grönke et al., 2010). Another research involves *Drosophila* insulin receptor mutants, characterized by a reduction

in IIS signaling with pleiotropic effects on many traits, including extreme dwarfism, sterility, increased fat levels, and shortened lifespan: interestingly, in presence of *Wolbachia* the IIS-related mutant phenotypes resulted in significant moderate effects (e.g., reduced fecundity and extended lifespan), suggesting that the symbiont acts to increase insulin signaling itself (Ikeya et al., 2009).

Wolbachia also seems to interact with *chico*, a gene encoding an insulin receptor substrate (Böhni et al., 1999). *Drosophila* carrying homozygous *chico*^{1} alleles are sterile, but in presence of *Wolbachia* the females produce progeny, even if significantly smaller than their heterozygous siblings (Clark et al., 2005; Richard et al., 2005).

Additional data on the possible interaction between the symbiont and host IIS are provided by studies on crustaceans. *Wolbachia* is known to infect several species of crustaceans, and especially (but not exclusively) terrestrial isopods where the symbiont can induce the feminization of genetical males through an interaction with host hormonal signaling pathways (Bouchon et al., 2008). Crustacea are by default females, and male sex differentiation is triggered by an androgenic hormone (AH) secreted by the androgenic gland (AG; Legrand et al., 1987; Sagi and Khalaila, 2001). The current hypothesis about the feminizing action of *Wolbachia* is that the symbiont interacts either with the AG differentiation process or the AH receptors (Rigaud and Juchault, 1998; Bouchon et al., 2008). However, if *Wolbachia* bacteria are experimentally inoculated in adult males, the host soon develops female structures, despite the presence of the AG, which becomes even hypertrophic (Martin et al., 1973, 1999). This suggests that the AH receptors are no longer functional, favoring the hypothesis that *Wolbachia* induces feminization by targeting the receptor of the AH. Interestingly, the AH has been proven to be an insulin-like peptide (Manor et al., 2007): *in vivo* silencing of the gene induces an arrest of spermatogenesis, prevents the regeneration of male secondary sexual characteristics, and also induces a lag in molt and a growth reduction (Ventura et al., 2009). In sequential hermaphrodites, the silencing of the AG insulin-like factor induces the feminization of male-related phenotypes too (Rosen et al., 2010).

WOLBACHIA AND ECDYSONE SIGNALING

Among invertebrates *Wolbachia* is known to infect exclusively (!) Arthropoda and Nematoda, two Phyla belonging to the Ecdysozoa, a clade of animals which share the ability to replace the exoskeleton. This process is called ecdysis and is controlled hormonally by a class of steroids called ecdysteroids (Ewer, 2005).

In filarial worms, antibiotic curing of *Wolbachia* "infection" inhibits nematode fertility and development, suggesting a specific role for the symbiont in host oogenesis, embryogenesis, and molting (Casiraghi et al., 2002; Arumugam et al., 2008; Frank et al., 2010). In arthropods, several data suggest that the phenotypic effects induced by *Wolbachia* may be linked to steroid-related processes. As it is well known, insect steroids play key roles in the coordination of multiple developmental processes, and in adults they control important aspects of reproduction.

In particular, during development, insect molting is induced by the systemic hormone 20-hydroxyecdysone (20E). 20E acts on members of an evolutionarily conserved family of nuclear receptors: it binds to the heterodimeric EcR/Usp receptor composed of EcR and USP (ultraspiracle, homologous to the vertebrate retinoid-X receptor), which shares many commonalities with the human thyroid hormone receptor. Then the EcR/USP complex activates the transcriptional processes underlying the cellular and morphogenetic molting cascade events (Gilbert et al., 2002). A biological action of 20E binding of un-partnered EcR has also been demonstrated (Spindler et al., 2009).

Despite the fact that ecdysteroids are present throughout the entire life of insects, their role in adults is quite elusive. Ecdysteroids, for example, may have a role in lifespan (Tricoire et al., 2009; Schwedes et al., 2011) or in stress responses, such as nutritional shortage, high temperature, dry starvation, and oxidative stress (Hirashima et al., 2000; Terashima et al., 2005; Ishimoto and Kitamoto, 2011). In adult flies ecdysone-mediated signaling is also involved in stressful social interactions and in homeostatic sleep regulation (Ishimoto and Kitamoto, 2011). Another unconventional role of the "molting" hormone is the control of important aspects of reproduction, including ovarian development and oogenesis (Raikhel et al., 2005). In many insect species 20E is directly involved in the regulation of vitellogenin biosynthesis by the female fat body, a metabolic tissue functionally analogous to the vertebrate liver; and it can also induce vitellogenin synthesis in males (Huybrechts and De Loof, 1977; Bownes et al., 1983; Zhu et al., 2007). The 20E has also been shown to affect sexual behavior (Ganter et al., 2007).

De Loof (2006) proposes that ecdysteroids may also act as sex hormones. In particular, 20E secreted by the follicle cells of the insect ovary could be the physiological equivalent of vertebrate estrogens, while E – the precursor of the active molting hormone 20E – should act as a distinct hormone, being the physiological equivalent of vertebrate testosterone (De Loof and Huybrechts, 1998; De Loof, 2006). Indeed, E can regulate a set of genes that are distinct from those controlled by 20E, thus confirming that it may exert different biological functions from 20E (Beckstead et al., 2007). However, in insects the existence of sex hormones is under debate, as the differentiation of primary and secondary sexual characteristics is generally considered under the exclusive control of the genotype of each single cell (Steinmann-Zwicky et al., 1989; Schütt and Nöthiger, 2000). However recent data demonstrate that in insects, as well as in vertebrates, non-autonomous (=hormonal) sex determination controls sex dimorphism (DeFalco et al., 2008; Casper and Van Doren, 2009). Thus, if ecdysteroids function as molting and sex hormones, this could explain why *Wolbachia* interferes with insect development and reproduction, as discussed in the following section.

Last but not least, it is worth noting that *Wolbachia* establish themselves in many host steroidogenic tissues, including the fat body and the ovarian follicular epithelium, as demonstrated in many insect species and shown in **Figure 1** (Sacchi et al., 2010; Gonella et al., 2011; Negri and Pellecchia, in press).

FEMINIZING *WOLBACHIA*: THE INDUCTION OF HOST FEMININE SEX DIFFERENTIATION DESPITE MASCULINE SEX DETERMINATION

Feminization, that is the development of genetical males into females, deals with sex differentiation much more directly than the other *Wolbachia*-induced phenotypes, thus offering the opportunity to shed light on the processes governing arthropod

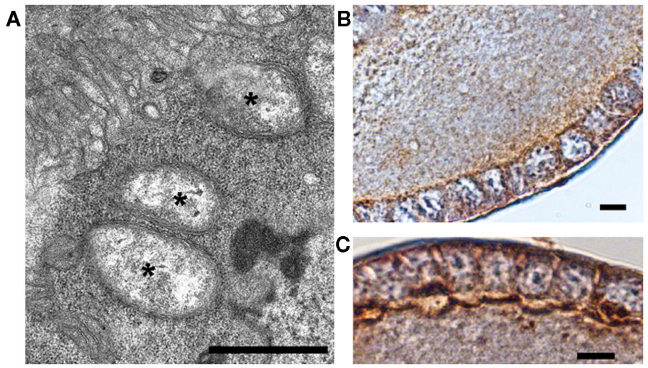

FIGURE 1 | *Wolbachia*'s localization in the host's follicular epithelium of the gonad. (A) TEM micrograph of a *Wolbachia*-infected *Zyginidia pullula* (Hemiptera, Cicadellidae) follicle cell filled with bacteria (asterisks; bar = 0.9 μm); **(B,C)** Immuno-histochemical reactions showing strong positivity (brown) to anti-*wsp* (*Wolbachia* surface protein) antibody in the leafhopper's follicular epithelium (bars = 10 μm). Modified from Negri and Pellecchia (in press).

sex development, and on the involvement of the endosymbiont in such processes.

Until now, in insects, feminization induced by *Wolbachia* has been demonstrated in lepidopteran and hemipteran species (Hiroki et al., 2002; Kageyama and Traut, 2003; Negri et al., 2006; Sakamoto et al., 2007). In the butterfly *Eurema hecabe*, it has been demonstrated that the *Wolbachia* feminizing effect acts continuously throughout the larval development for the maintenance of the female phenotype (Narita et al., 2007). Accordingly, this suggests that the bacterium acts on the insect sex differentiation rather than sex determination, and ecdysteroids are the best candidate for such an interaction. Evidences are in fact provided by the effects of incomplete *Wolbachia* suppression by antibiotic treatments during lepidopteran larval stages. In particular some tetracycline-treated individuals show larval/pupal molting defects, and others do not pupate: the dissection of dead pupae reveals that many of them failed to escape from the pupal case (Narita et al., 2007). Similar molting defects may be obtained, for example, in ecdysone receptor knock-out individuals of *Blattella Germanica* and *D. melanogaster* EcR-mutants (Davis et al., 2005; Cruz et al., 2006). Moreover, antibiotic treatments in infected *E. hecabe* often induce also sexually intermediate traits in wings, gonads, and genitalia (Narita et al., 2007). Notably, in Lepidoptera a role for the ecdysteroid titer in regulating sexual dimorphism, including sex specific wing development, has been proved (Lobbia et al., 2003) strengthening the hypothesis of a link between *Wolbachia* and ecdysteroid signaling. In particular, we cannot exclude a host/symbiont co-adaptation where a partial symbiont removal leads to biological imbalance. This may also explain the origin of intersex individuals in *E. hecabe*, *Ostrinia scapulalis*, and *O. furnacalis* partially cured by feminizing *Wolbachia* (Kageyama and Traut, 2003; Sakamoto et al., 2007). Intersexes are specimens characterized by a genetically homogeneous genotype (male in this case), but a mixture of male and female phenotypes (i.e., feminized tissues in this case): the appearance of these phenotypes may be the result of a partial but evident conflict between male and female sex hormones and/or receptors.

In *Ostrinia* species, in addition, a complete feminization is fatal and genetical males die during the larval development (Kageyama and Traut, 2003; Sakamoto et al., 2007), while in other species the male-killing action of *Wolbachia* occurs during embryogenesis (Werren et al., 2008). As discussed above, the role played by ecdysteroids during the whole developmental cycle of insects is crucial. Embryogenesis, in particular, takes place in a steroid hormone-enriched environment, where steroids act for the coordination of morphogenetic movements (Kozlova and Thummel, 2003; Gaziova et al., 2004). Thus, if male-killing *Wolbachia* interacts with the host hormonal pathway involving ecdysteroids, this could affect the processes required for a normal development of males.

A sex-specific action of ecdysteroids during insect embryogenesis and development has been demonstrated in some studies concerning the effects of endocrine disrupting chemicals. Indeed, ecdysteroid agonists and antagonists (e.g., bisphenol A, tebufenozide, and ethinyl estradiol) are responsible for female-biased sex ratios in the treated populations (Hahn et al., 2001; Biddinger et al., 2006; Lee and Choi, 2007; Izumi et al., 2008). According to some authors, the observed sex-specific effect could be well explained by considering insect steroids as sex hormones. In particular, larval or embryo males die because they are subjected to an unsuitable, i.e., female, hormonal environment (Hahn et al., 2001).

WOLBACHIA, HOST OOGENESIS DEFECT RESCUING AND FECUNDITY ENHANCEMENT

In some cases *Wolbachia* is essential for insect reproduction, as in absence of the "infection" the host is not able to perform a normal oogenesis. For example, in the hymenopteran *Asobara tabida* the symbiosis with *Wolbachia* involves interference with the programmed cell death of nurse cells that is significantly higher in *Wolbachia*-cured insects (where the ovary completely lacks mature oocytes) than in naturally infected specimens (Dedeine et al., 2001; Pannebakker et al., 2007). The role of ecdysone in regulating cell apoptosis, a process required for insect development, is well known: during metamorphosis, for example, the steroid is a primary regulator of cell death in larval tissues which are destroyed or remodeled into an "adult" form (Tsuzuki et al., 2001; Mottier et al., 2004). Interestingly, in adults a higher level of 20E causes apoptosis of nurse cells which blocks the oogenesis process (McCall, 2004; Terashima et al., 2005; Ishimoto and Kitamoto, 2011). Therefore, it would be interesting to verify if *Wolbachia* interacts by modulating ecdysone signaling in *A. tabida*, thus influencing programmed cell death pathways occurring during host oogenesis.

In *D. melanogaster*, partial loss of function mutants of sex-lethal, the master regulator gene of the fly sex determination cascade, are sterile due to overproliferation of undifferentiated germ cells. In the infected line, *Wolbachia* is able to rescue oogenesis defects leading to partially fertile specimens (Starr and Cline, 2002). Stem cell behavior is regulated by intrinsic factors, signals from their niches and systemic hormones. Ecdysone is known to affect stem cell proliferation, also confirming current hypotheses

of an involvement of steroids in cancer: in particular, altered steroids signaling, as well as extensive molecular crosstalk between steroid and insulin/insulin-like growth factors, are commonly associated with cancer (Ables and Drummond-Barbosa, 2010). Accordingly, we may speculate that the occurrence of "cancer" germ cells could be due to a misregulation of ecdysone signaling in mutant flies that is rescued by *Wolbachia* infection.

Wolbachia has also been implicated in improving the fitness of several insect hosts (Dedeine et al., 2003).

New insights into the mechanisms underlying host fecundity enhancement are provided by a recent study on *D. mauritania*: *Wolbachia* improves fecundity both by enhancing stem cell proliferation and reducing programmed cell death in the germarium (Fast et al., 2011). The authors hypothesize that the presence of the bacterium in the germ-line stem cell niche modulates stem cell activity, although a contribution from systemic or stem cell intrinsic signals cannot be ruled out. It remains unclear, however, whether stem cells themselves sense and respond to *Wolbachia*. According to us, the role of ecdysone might be of primary importance, as the systemic hormone is known to stimulate directly germ-line stem cells in order to promote their self-renewal and activity (Ables and Drummond-Barbosa, 2010), with *Wolbachia* as the director of the scene.

INTERPLAY BETWEEN STEROID SIGNALING AND EPIGENETIC PATHWAYS

A growing body of data suggests a role for hormones in modulating epigenetic changes. In mammals, for example, steroids are able to induce epigenetic differences necessary for a correct sex differentiation of the brain (Nugent and McCarthy, 2011), and in adults they actively maintain DNA methylation patterns (Auger et al., 2011). An interaction between steroid/thyroid receptors and the epigenetic machinery (e.g., histone modifying enzymes and DNA methyltransferases) has been proposed too (Tsai et al., 2009; Haddad et al., 2010; Pathak et al., 2010). Novel insights into the mechanisms underlying such an interaction are provided by studies on nuclear receptor co-regulators (NRCs; i.e., co-activators and co-repressors). Strikingly, NRCs are key epigenetic regulators and utilize enzymatic activities to modify epigenetically the DNA and chromatin (Mahajan and Samuels, 2000; Rosenfeld et al., 2006; Hsia et al., 2010).

Thanks to studies on *Drosophila*, we now have compelling evidences of a direct interaction between steroids (specifically ecdysone) and epigenetic factors. For example, during fly development, neural circuit sculpting is due to cooperation between EcR and histone modifying enzymes (Kirilly et al., 2011). In addition, in the fly's ovary, ecdysone interacts with chromatin remodeling factors for modulating the proliferation and self-renewal of germline stem cells (Ables and Drummond-Barbosa, 2010). Ecdysone receptor signaling also needs direct cooperation with nucleosome remodeling complexes, and many EcR co-activators and co-repressors that contribute to the epigenetic memory have been identified and characterized (Kimura et al., 2008; Sawatsubashi et al., 2010; Kugler et al., 2011).

Interestingly, recent data demonstrate that *Wolbachia* infection is able to modulate the host genomic imprinting through methylation of the DNA (Negri et al., 2009a,b). In the leafhopper *Z. pullula*, *Wolbachia*-infected genetic males develop into intersexes with a female phenotype. In particular, two kinds of intersexes are described: "intersex females" which are feminized males with ovaries, even able to produce progeny; and "intersex males" which bear testes and are characterized by a very low *Wolbachia* density (Negri et al., 2009a). Remarkably, *Wolbachia*-infected "intersex females" possess the same imprinting pattern of uninfected females, thus demonstrating that the infection disrupts the male imprinting (Negri et al., 2009a,b). In addition, the alteration occurs only if the bacterium exceeds a density threshold, as "intersex males" maintain a male genome-methylation pattern (Negri et al., 2009a). The epigenetic modifications affect the expression of genes involved in sex determination and development (Negri, unpublished data), thus avoiding the need for *Wolbachia* to interfere with each single gene separately.

In view of the interplay between hormone signaling and epigenetic machinery, data on the whole suggest that the manipulation of the host's epigenetic pathways might be achievable through a direct influence of *Wolbachia* on hormonal signaling involving ecdysteroids.

The model proposed in **Figure 2** tries to explain possible interactions. Once 20E is biosynthesized, it binds the nuclear receptor EcR which heterodimerizes with USP. The EcR/USP complex binds DNA and complexes with nuclear NRCs. Then, NRCs catalyze DNA methyltransferases for a correct methylation pattern of differentially methylated regions or directly function as histone modifying enzymes, thus activating proper selective transcriptional programs. In infected insects, *Wolbachia* may interact with the ecdysone pathway by synthesizing products competing with 20E or function as/interfere with NRCs. As a result, the EcR binding to DNA or the recruitment of DNA methyltransferases and/or histone modifying enzymes should be affected.

Accordingly, studies on *Wolbachia*–host interactions should give great attention for example to substances with an antagonist action on the ecdysone nuclear receptor; selective nuclear receptor modulators; or co-regulators of nuclear receptors, in view of their emerging role in integrating the transcriptional co-regulation with the epigenetic regulation (Rosenfeld et al., 2006; Kato et al., 2011).

CONCLUSION

Many experimental data support the role of *Wolbachia* in modulating the insect sexual phenotypes by interaction with the host hormonal signaling pathway. Indeed, the various phenotypic effects observed may be due to differences in host physiology and in particular to endocrine-related processes governing growth, development, and reproductive behavior.

In particular, a number of evidences links *Wolbachia* symbiosis to insulin and ecdysteroid signaling.

Several studies report an extensive crosstalk between the two hormonal signaling pathways, which may work antagonistically, jointly or even independently. Like many other symbiotic bacteria, *Wolbachia* could operate by attacking one crucial pathway in their hosts, thus affecting other metabolic networks, or by targeting both pathways, even interacting at several points in each of them for its own benefit (that is the manipulation of host reproduction and development in order to increase the number of infected females). In view of the interplay between hormone signaling and epigenetic

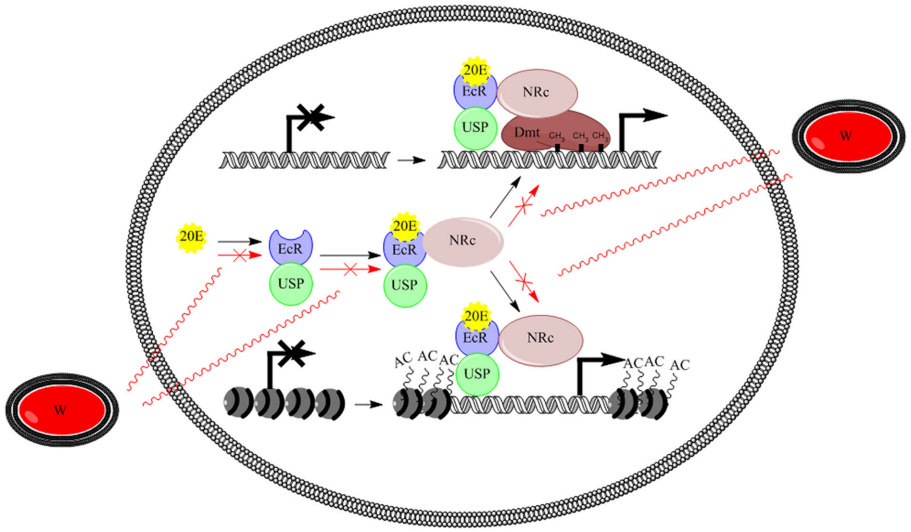

FIGURE 2 | Model illustrating the possible interplay between ecdysone signaling and epigenetic regulation, and *Wolbachia* action. 20E binds the nuclear receptor EcR which heterodimerizes with USP. Then, the EcR/USP complex binds DNA and complexes with nuclear receptor co-regulators, which catalyze DNA methyltransferases or directly function as histone modifying enzymes, thus activating proper selective transcriptional programs. *Wolbachia* may interact by synthesizing products competing with 20E or function as/interfere with nuclear receptor co-regulators, respectively. 20E, 20-hydroxyecdysone; EcR, ecdysone receptor; USP, ultraspiracle; NRc, nuclear receptor co-regulator; Dmt, DNA-methyltransferase; W, *Wolbachia* bacteria

machinery, a direct influence of the "infection" on hormonal signaling involving ecdysteroids might be achievable through the manipulation of host epigenetic pathways.

Although further work is needed to fully clarify the genetic and molecular bases of such an interaction, new work hypotheses have been now offered for the study of the mechanisms used by symbionts to dialog with their hosts. Likewise, the *Wolbachia*–host interaction should become an emerging model system for the study of hormone signaling orchestration made by microbial symbionts playing with nuclear receptors, and for shedding light on the role of NRCs in integrating the transcriptional co-regulation with the epigenetic regulation.

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