Sex and stripping
The key to the intimate relationship between Wolbachia and host?

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Wolbachia pipientis is known to infect only arthropods and nematodes (mainly filarial worms). A unique feature shared by the two Phyla is the ability to replace the exoskeleton, a process known as ecdysis. This shared characteristic is thought to reflect a common ancestry. Arthropod moulting is induced by the steroid hormone 20-hydroxyecdysone (20E) and a role for ecdysteroids in nematode ecdysis has also been suggested. Removing Wolbachia from filarial worms impairs the host’s development. From analyses of the genome of Wolbachia harbored by the filarial nematode Brugia malayi and that of its host, the bacterium may provide a source of heme, an essential component of cytochrome P450’s that are necessary for steroid hormone biosynthetic pathways. In arthropods, Wolbachia is a reductive manipulator, inducing various phenotypic effects that may be due to differences in host physiology, in particular, endocrine-related processes governing development and reproduction. Insect steroids have well-defined roles in the coordination of multiple developmental processes, and in adults they control important aspects of reproduction, including ovarian development, oogenesis, sexual behavior, and in some taxa vitellogenin biosynthesis. According to some authors ecdysteroids may also act as sex hormones. In insects sex determination is generally thought to be a strictly genetic process, in which each cell decides its own sexual fate based on its sex chromosome constitution, but, surprisingly, recent data demonstrate that in Drosophila sex determination is not cell-autonomous, as it happens in mammals. Thus the presence of signals coordinating the development of a gender-specific phenotype cannot be excluded. This could explain why Wolbachia interferes with insect reproduction; and also could explain why Wolbachia interferes with insect development.

Thus, is “sex (=reproduction) and stripping (=ecdysis)” the key to the intimate relationship between Wolbachia and its host?

Introduction

Wolbachia is an intracellular alphaproteobacterium, and is widespread in arthropods and in nematodes, mainly in filariae.1 In both types of hosts, this bacterium is vertically transmitted from mother to offspring. Wolbachia is well known for its ability to manipulate the reproduction of arthropod hosts, while in filarial nematodes this bacterium is regarded as an obligate symbiont. Indeed, antibiotic treatments targeted toward Wolbachia impair the fertility of female adult filariae and block the development of the larvae, suggesting a role for Wolbachia in the oogenesis, embryogenesis and moulting of the host nematodes.2-4 Moulting, i.e., the ability to replace an exoskeleton, is a unique characteristic, shared by Arthropoda and Nematoda. High level phylogenies group these two phyla into the Ecdysozoa clade.5 The fact that only arthropods and nematodes have thus far been found to harbour Wolbachia might thus reflect some hidden similarities in the intimate relationship between Wolbachia and its hosts. Insect moulting is induced by the steroid hormone 20-hydroxyecdysone (20E), whose precursor is secreted by prothoracic glands after their stimulation by the brain prothoracotropic hormone (PTTH). Dietary cholesterol is converted to 20E thanks to hydroxylation reactions catalyzed by cytochrome P450 enzymes. 20E binds the heterodimeric nuclear receptor EcR/Usp (Ecdysone Receptor/Ultraspireacle), and activates the transcriptional processes underlying the cellular and morphogenetic moulting cascade events (Fig. 1).6

In nematodes, as well, moulting seems to be regulated by ecdysteroid-like hormones and orthologs of insects nuclear receptors, involved in ecdysone response, have been found.7,8 In Caenorhabditis elegans these nuclear receptors are also involved in the regulation of sex determination and reproductive development.9,10 Interestingly, in filarial worms the removal of Wolbachia causes various detrimental effects on the host, which suffers impaired larval development.11, 12 Combined analyses of the genomes of Wolbachia and its filarial host Brugia malayi7,13 suggest that the bacterium provides the nematode with heme, the prosthetic group of enzymes required for a variety of functions, including the biosynthesis of ecdysteroid hormones.4,14

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In arthropods Wolbachia can induce cytoplasmic incompatibility, parthenogenesis, feminization, male-killing, fecundity enhancement, and even rescue of oogenesis defects. Such phenotypic variability is thought to be linked to high genome plasticity of insect-borne Wolbachia, since all of the genomes thus far sequenced for these symbionts contain high number of repetitive sequences, including IS (insertion sequences) elements and prophage-like sequences.

We argue that the various phenotypic effects observed in insects may be due to differences in host physiology, and in particular to endocrine-related processes governing development and reproduction which in insects display high variability. Insect steroids have a well-defined role in the coordination of multiple developmental processes, and in adults they control important aspects of reproduction, including ovarian development, oogenesis, and, in some taxa, vitellogenin biosynthesis. In addition, recent data suggest a role for ecdysone and its receptors in sexual behavior.

According to De Loof ecysteroids may act as sex hormones. In insects sex differentiation is generally thought to be a strictly genetic process, in which each cell decides its own sexual fate based on its sex chromosome constitution, but recent data demonstrate that, as in mammals, in the fruit fly Drosophila melanogaster non-autonomous sex determination controls sex dimorphism. Thus the presence of signals coordinating the development of a gender-specific phenotype cannot be excluded.

By carefully examining the available literature, in the following sections we propose a new perspective, supporting the role of Wolbachia in modulating the insect host sexual phenotypes by interaction with hormonal pathways, as it has already been shown for isopod crustaceans.

Feminization

Feminization is a phenomenon in which genetic male embryos carrying Wolbachia bacteria develop into females. In arthropods, feminization was first described in isopod crustaceans, including Armadillidium vulgare, Oniscus asellus and Porcellionides pruinosus. Later, feminization among insects was demonstrated in the Lepidopteran Eurema hecabe and in the Hemipteran Zyginidia pullula. In all these cases, the existence of intersexes has been described, and the feminizing effect was modulated by bacterial cell density.

Authors generally state that in insects sex determination and differentiation are strictly genetic. This is supported by the observation that in Drosophila and the silkmoth Bombyx mori female specific genes are only expressed by female cells of gynandromorphs, aberrant specimens made up of both female cells (XX in Drosophila and ZW in B. mori, respectively) and male cells.

Figure 1. Insect moulting is induced by the circulating steroid hormone 20-hydroxyecdysone (20E) whose biosynthesis takes origin from dietary cholesterol. The process is initiated by secretion of the brain neuropeptide prothoracicotropic hormone (PTTH). PTTH acts on the prothoracic glands, triggering the synthesis and secretion of a pre-ecdysteroid which is sequentially converted to active 20E. Cholesterol is converted to 20E thanks to a number of hydroxylation reactions catalysed by cytochrome P450 enzymes of microsomal and/or mitochondrial origin. Then, 20E binds heterodimeric nuclear receptors composed of EcR (Ecdysone Receptor) and Usp (Ultraspiracle), and activates the transcriptional processes underlying the cellular and morphogenetic moulting cascade events. Even if PTTH is the the major tropic hormone driving ecdysone secretion, also insulin-like peptides (ILPs) may induce ecdysteroidogenesis, through stimulation of the prothoracic gland growth. BR (brain); ILPs (insulin-like peptides); PTTH (prothoracicotropic hormone); PG (prothoracic gland); EcR/USP (Ecdysone Receptor/Ultraspiracle).
(XY in Drosophila and ZZ in B. mori, respectively). How can one explain the existence of intersexes, i.e., individuals with an intermediate phenotype between male and female but genetically homogeneous pattern, if each cell makes its own sexual decision? If there were signals coordinating the development of a gender-specific phenotype, intersexes might arise from imbalance or conflict between male and female sex hormones and/or receptors. This has been confirmed by Rigaud and Juchault in the isopod A. vulgare, where intersexes are the result of a conflict between male differentiation and bacterial feminizing action.

In E. hecabe, Narita et al. found that feminizing Wolbachia acts continuously throughout the larval development to produce the female phenotype. Since in lepidopteran sex determination is probably completed very early in embryogenesis, the authors speculate that the feminizing Wolbachia may interact with some female- or male-specific molecular mechanisms that are located downstream of the sex determination system and are responsible for the expression of female-specific phenotypes. If there were signals coordinating the development of a gender-specific phenotype, intersexes might arise from imbalance or conflict between male and female sex hormones and/or receptors. This has been confirmed by Rigaud and Juchault in the isopod A. vulgare, where intersexes are the result of a conflict between male differentiation and bacterial feminizing action.

Interestingly, the AH was found to be a member of the evolutionarily related insulin and/or insulin-like growth factor families. This peptidic hormone consists of two chains linked by disulfide bridges, one of them glycosylated. The mature hormone is derived from a precursor after excision of a C peptide, following maturation steps similar to the ones involved in insulin synthesis processes. These characteristics including proteolytic cleavage motifs and conserved cysteine residues seem to be a common feature of all isopod AHS studied so far (Grève P, unpublished data).

Studying the crayfish Cherax quadricarinatus, Manor et al. point out that even if insulin and hormones members of the insulin family are generally not regarded as gender-specific, their expression in the crustacean AG is gender-specific, suggesting that they may have evolved in the context of regulating sexual differentiation. In fact, the crustacean AG regulates several sex-related phenomena, such as male-like aggressive and reproductive behaviors, and even differential growth rate between male and female. In the shrimp Macrobrachium rosenbergii, in vivo silencing of the gene encoding an insulin-like AG peptide led to the arrest of testicular spermatogenesis accompanied by hypertrophy of the AGs and prevented the regeneration of male secondary sexual characteristics, but also included a lag in molt and a reduction in growth parameters.

Insulin-like peptides (ILPs) are also found in insects. Bombyxins, a family of peptides produced by the brain of B. mori, were first of these found to have a structural homology to vertebrate insulins. Subsequent studies on Drosophila homologs revealed the existence of multigene families, expressed in insect brain and other tissues. The ILPs are among the major products revealed the existence of multigene families, expressed in insect brain and other tissues. The ILPs are among the major products synthesized by the median neurosecretory cells of the insects’ brain and may thus be considered as true neurohormones. ILPs regulate diverse functions, including growth, metabolism, fecundity and lifespan. Moreover some ILPs are predicted to be more similar to insulin-like growth factors (IGFs) than to insulin. They have distinct domain organizations and physiological functions, and they also differ in the mode of secretory regulation: for example, IGFs-like peptides are predominantly produced by the fat body, a functional equivalent of the vertebrate liver and adipocytes. Interestingly, ILPs have an indirect effect on insects’ steroidogenesis through the stimulation of the gland growth. In fact, two ecdysteroidogenetic axes are known in insects, the brain-prothoracic gland axis involved in development, and the brain-gonad axis involved in reproduction. In the first axis, ecdysteroid synthesis is triggered not only by prothoracotropic hormone (PTTH), but also by insulin-like peptides (ILPs), secreted by insect brain. In the second axis, ecdysteroid synthesis in the gonads (e.g., follicular cells in ovaries) is triggered by gonadotropins and, again, ILPs.

In Drosophila the chico gene encodes a major insulin receptor substrate involved in growth regulation. Mutations in chico impair the proliferation of ovarian follicle cells and block egg chamber progression into vitellogenesis. Thus, the mutation...
blocks the insulin signalling needed for yolk protein synthesis and uptake in the ovary. Homozygous chico females are sterile, but in presence of Wolbachia these mutant lines produce progeny. Thus, Wolbachia action on hormonal pathways involving ecdysteroidogenesis could circumvent this insulin pathway defect (i.e., sterility).

Moreover, a recent study on Drosophila mutants for insulin/IGF-like signaling (IIS) (individuals characterized by moderate dwarfism, reduced fecundity and extension of female lifespan) indicates that Wolbachia may increase insulin signaling. In fact, symbiont removal further reduces IIS, enhancing IIS-related phenotypes such as extreme dwarfism, sterility, increased fat levels and shortened lifespan.

**Male-Killing**

Among the reported cases of male-killing Wolbachia, those of *Ostrinia scapulalis* and *O. furnacalis* are particularly intriguing. In these two moths Wolbachia kills genetic males (ZZ) during the larval stage, while genetic females (WZ) do not survive in absence of the bacterium. As in the *Eurema hecabe* feminization case, incomplete curing of male-killing Wolbachia infection produces *Ostrinia* intersexes. The authors considered these individuals partly feminized males, whereas complete feminization seemed to be incompatible with the survival of the male genotype. This leads to the hypothesis that male-killing is just an unsuccessful “attempt” at feminization by Wolbachia. Is this another density-dependent effect induced by the bacterium on the host hormonal system during development and sex differentiation?

In all these cases, sex-specific killing action by Wolbachia occurs during host development; in others, male-killing occurs during embryogenesis. It should be noted that embryogenesis takes place in a steroid hormone-enriched environment, and steroid hormones act for the coordination of morphogenetic movements.

In Drosophila ecdysteroid levels begin to rise during early embryogenesis around the time of gastrulation and peak at stage 11–12 during germ band retraction (GBR), which is one of the major morphogenetic movements shaping the body plan of the first instar larva. This happens about the time when the expression of genes shadow (sad) and disembodied (dib), coding for key enzymes of ecdysone biosynthesis (i.e., P450 cytochromes), show a striped pattern.

The only observations available on male embryonic death mechanisms involve Drosophila and the male-killer bacterium Spiroplasma: male embryonic development arrests before segmentation, just around the point of GBR. Thus, if male-killing Wolbachia interacts with the ecdysteroidogenic pathways, this could interfere with the spatial and temporal expression patterns of cytochrome P450s required for a normal development of males.

Unfortunately, little information about sex-specific action of ecdysteroids during embryogenesis and development in insects is available, and it mainly concerns the effects of endocrine-disrupting chemicals. However, in vertebrates several studies on endocrine disruption indicate that some chemicals may have sex-specific effects, effects on sex differentiation and alterations in the sex ratio. Indeed, in the housefly *Musca domestica* the sex ratio was one of several phenotypes to be affected by bisphenol A, a chemical that interferes with ecdysteroid-dependent physiological processes. In particular, the sex-ratio shifted toward males when eggs and larvae were exposed to the chemical treatment. Similar results were obtained on larvae of the moth *Plutella xylostella* after treatment with the molting-hormone agonist tebufenozide, but tebufenozide treatment on larvae of the midge *Chironomus riparius* produced a female-biased sex ratio. According to the authors, the observed sex-specific effect could be explained by considering insect steroids sex hormones, with 20-hydroxyecdysone (20E) as the insect equivalent of vertebrate estrogen and its precursor ecdysone (E) as the insect equivalent of vertebrate testosterone. According to this view, in *C. riparius* tebufenozide might simulate higher 20E (i.e., female hormone) and consequently lower E levels (i.e., male hormone), when 20E binds to the ecdysone receptors. The likely induction of enzymes that metabolize E into 20E may lead to a further decrease in the E titer. This combination might affect male pupae more than female, so that male pupae die because they are subjected to an unsuitable, i.e., female, hormonal environment.

**Conclusion**

An interaction between Wolbachia and host hormonal pathways involving ecdysteroids may suggest the mechanistic way the bacteria uses for manipulating the host’s sexual behavior, opening new work hypothesis for the study of the evolutionary interactions between bacteria and host.

In all the known Wolbachia/host systems, the mechanisms underlying the interaction should be the same and the various phenotypic effects observed may be due to differences in host physiology, considering that endocrine-related processes governing host development and reproduction display an enormous variability.

We may also speculate that the various phenotypic effects induced by Wolbachia reflect an evolution underway between the symbiont and its host. In particular, effects on the host may range from negative to positive, as Wolbachia may be a reproductive manipulator, a facultative or an obligate symbiont, where it becomes essential for host survival or host fertility.

Theory predicts that maternally-transmitted endosymbions will be selected towards mutualism, increasing the fecundity of their female hosts. Detrimental effect on its host should result in selection for the bacteria to evolve a more benign lifestyle, changing the bacterium from being parasitic to more mutualistic. Shifts from reproductive manipulator to beneficial symbiont, i.e., from an “imperfect feeling” between Wolbachia and its host to a more perfect one, are documented and can also be rapid. A further clue of this dynamic association might be the substantial variation in Wolbachia density among individuals of the same population, as observed in feminization, male-killing and cytoplasmic incompatibility, where the induced phenotype is positively correlated with the bacterium density.

Thus, is sex and stripping the key to the (mostly) “unsteady” intimate relationship between Wolbachia and its host?
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