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Unravelling the *Wolbachia* evolutionary role: the reprogramming of the host genomic imprinting

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Environmental factors can induce significant epigenetic changes that may also be inherited by future generations. The maternally inherited symbiont of arthropods *Wolbachia pipientis* is an excellent candidate as an 'environmental' factor promoting trans-generational epigenetic changes: by establishing intimate relationships with germ-line cells, epigenetic effects of *Wolbachia* symbiosis would be manifested as a 'maternal effect', in which infection of the mother modulates the offspring phenotype. In the leafhopper *Zyginidia pullula*, *Wolbachia* feminizes genetic males, leaving them as intersexes. With the exception of male chitinous structures that are present in the last abdominal segment, feminized males display phenotypic features that are typical of females. These include ovaries that range from a typical histological architecture to an altered structure. Methylation-sensitive random amplification of polymorphic DNA profiles show that they possess a female genomic imprint. On the other hand, some rare feminized males bear testes instead of ovaries. These specimens possess a *Wolbachia* density approximately four orders of magnitude lower than feminized males with ovaries and maintain a male genome—methylation pattern. Our results indicate that *Wolbachia* infection disrupts male imprinting, which dramatically influences the expression of genes involved in sex differentiation and development, and the alteration occurs only if *Wolbachia* exceeds a density threshold. Thus, a new *Wolbachia*'s role as an environmental evolutionary force, inducing epigenetic trans-generational changes, should now be considered.

Keywords: *Wolbachia pipientis*; *Zyginidia pullula*; male feminization; bacterium density; DNA methylation; histology

1. INTRODUCTION

Epigenetic changes are based on molecular mechanisms including methylation of cytosines, remodelling of chromatin structure through histone chemical modifications and RNA interference.

The ability of environmental factors to induce epigenetic changes is currently one of the most active emerging topics in biology. Studying monozygotic twins, i.e. natural human clones, a recent work demonstrated the significant role of environmental factors in translating a common genotype into different phenotypes (Fraga *et al.* 2005). Remarkably, environmentally induced epigenetic traits can be inherited by future generations, despite the presence of whole-genome reprogramming systems (Reike & Walter 2001). Notwithstanding these barriers, trans-generational epigenetic inheritance is not a rare phenomenon, leading the scientific community to investigate the 'etiological' role of epigenetics in disease, and to exploit the possibility of creating epigenetic tumour-inhibiting treatments (Cropley *et al.* 2006; Richards 2006).

The alpha-proteobacterium *Wolbachia pipientis* is a widespread, maternally inherited endosymbiont of arthropods that manipulates the reproduction of its hosts to increase the proportion of infected females. *Wolbachia pipientis* is responsible for cytoplasmic incompatibility between gametes, skews sex ratios towards females in a variety of ways (i.e. male killing, feminization and parthenogenesis) and even rescues host oogenesis defects (as reviewed in Stouthamer *et al.* 1999). Maternal transmission is accomplished via incorporation of *Wolbachia* into female germ-line cells. In *Drosophila*, bacteria are present in germ-line stem cells or may reach oocytes through the somatic stem cell niches (Frydman *et al.* 2006; Serbus *et al.* 2008). During embryogenesis, they became incorporated into the pole cells, the precursors of germ-line stem cells (Serbus & Sullivan 2007). This behaviour makes *Wolbachia* an excellent candidate as an 'environmental' factor that promotes heritable epigenetic changes in host gene expression. The potential epigenetic effects of *Wolbachia* symbiosis would be manifested as a 'maternal effect', in which infection of the mother alters the offspring phenotype.

In the grass-dwelling European leafhopper *Zyginidia pullula* (Boheman; Hemiptera, Cicadellidae), *Wolbachia* feminizes genetic males, leaving them as intersex females, i.e. specimens with an external morphology typical of a female, except for an irregular anogenital zone that maintains male chitinous structures (Negri *et al.* 2006). To date, *Wolbachia*-induced male feminization has been reported in the lepidopteran species *Eurema hecabe*, *Ostrinia scapulalis* and *Ostrinia furnacalis* (Hiroki *et al.*

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2002; Kageyama & Traut 2004; Sakamoto *et al.* 2007). In the *Ostrinia* species, *Wolbachia* kills genetic males after an unsuccessful 'attempt' at feminization (Kageyama & Traut 2004; Sakamoto *et al.* 2007). Lepidoptera and *Z. pullula* have different sex determining chromosomes, since the Lepidoptera possess a ZZ/ZW sex chromosome system, with females as the heterogametic sex (Hiroki *et al.* 2002; Kageyama & Traut 2004), while *Z. pullula* has an XX/X0 model, with XX females and X0 males (Negri *et al.* 2006). These data, as a whole, suggest that *Wolbachia* could interact with the genetic control system involved in sex determination and differentiation, independently of the sex chromosome system.

The mechanism used by *Wolbachia* for altering host reproduction is not clear, and in this work we provide, to our knowledge, the first evidence that this symbiont interferes with the host genetic imprinting, altering the methylation pattern of the leafhopper genome. Furthermore, we present histological results that are, to our knowledge, the first published data on gonad morphological organization in males feminized by *Wolbachia*.

2. MATERIAL AND METHODS

(a) *Sample collection and rearing*

Zyginidia pullula females were sampled in the province of Parma (northern Italy) and reared individually so as to obtain isofemale lines. The *Wolbachia* infection was assessed on parental females by PCR using the V1–V6 primers targeting the *Wolbachia*'s 16S rRNA gene, as previously described (Negri *et al.* 2006).

(b) *Methylation-sensitive random amplification of polymorphic DNA PCR*

DNA samples were extracted from single *Z. pullula* adults (10 males, 10 females and 8 feminized males) and from adult gonads (18 males, 18 females and 8 feminized males) using the Wizard Genomic DNA Extract kit (Promega, Fitchburg, WI, USA) or DNAeasy Blood & Tissue Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. DNA samples were quantified using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE, USA).

Digestion of genomic DNA with the *Msp* I and *Hpa* II (Fermentas, Hanover, MD, USA) endonucleases was performed at 37°C for 16 hours in the appropriate buffers.

Methylation-sensitive random amplification of polymorphic DNA (RAPD) PCR was performed using the primers OPA1 (5′- CAGGCCCTTC), OPA3 (5′-AGT CAGCCAC), OPA4 (5′-AATCGGGCTG) and RAPD 600 (5′-GAAGAACCGC) using genomic DNA digested with *Msp* I and *Hpa* II as templates. RAPD PCRs have been carried out using DNA extracted from single samples, repeating each PCR amplification five times with each primer in order to verify the consistency of the results. All the PCR amplification mixtures contained 80 ng of digested genomic DNA, 1 μM of primer, 200 μM dNTPs and 2 U of DyNAZyme II polymerase (Finnzymes Oy). Amplifications were carried out by using a thermal cycler Hybaid Omn-E (Hybaid, Franklin, MA, USA) with the following parameters: 94°C for 2 min; 35 cycles at 93°C for 1 min; 36°C for 1 min; 72°C for 3 min and 30 s; and a final extension at 72°C for 7 min. PCR products were subjected to agarose gel (1.2% [w/v]) electrophoresis in 1×TAE buffer. DNA was stained with ethidium bromide and photographed under UV light.

(c) *Gonad histological characterization*

Gonads of uninfected females and males, and gonads of 50 *W. pipientis*-infected intersexes were dissected, fixed in Bouin's mixture and embedded in agar/paraffin wax, as described by Franchini *et al.* (2003). To inspect the anogenital zone, the pygofer removed from feminized males was prepared on a microscope slide in a lactic acid droplet. Haematoxylin/eosin histological stain and gallocyanin–chrome alum histochemical reaction were applied on 7 μm serial sections to study the gonadic structural organization.

(d) *Quantitative PCR*

Quantitative PCR (qPCR) was used to measure the abundance of *Wolbachia ftsZ* DNA and of *Z. pullula*'s 18S rDNA gene, as previously described (Noda *et al.* 2001; McGarry *et al.* 2004). qPCRs were performed using whole specimens, including gonads that have been previously dissected for checking the individual sex. The reactions were performed with an I-cycler thermal cycler (Bio-Rad, Richmond, CA, USA) using primers targeting the *Wolbachia ftsZ* DNA: Zp*ftsZ*Fw (5′-ACTGGTGGCGGA GATATGAF-3′) and Zp*ftsZ*FwR (5′-TCCATCGCCTGAT CAAAAGT-3′); and primers on the insect's 18S rDNA gene: 18S1F (5′-TACCTGGTTGATCCTGCCAGTAG-3′) and 18SdelR1 (5′-AATTTGTTCAAAGTAAACGTGCCGG-3′) (Cryan 2005). The reactions were performed with Brilliant SYBR Green qPCR Master Mix (Stratagene, La Jolla, CA, USA), with the following parameters: 94°C for 3 min; 50 cycles at 94°C for 45 s; 54°C (for *ftsZ*) or 57°C (for 18S rDNA) for 1 min; and 72°C for 1 min. Fluorescence data were collected during each cycle at 72°C. For qPCRs, a final step for the melting curve analysis from 70 to 95°C, measuring fluorescence every 0.5°C, was added; in addition a sample of products was sequenced, and sequences have been deposited in GenBank under the accession numbers EU742615 and EU742616 for 18S rDNA and *ftsZ*, respectively. Copy numbers in the starting templates were calculated by reference to the standard curves, obtained by using plasmids containing inserts of the target sequences cloned with pGEM T Easy Vector Cloning Kit (Promega) and quantified for copy number calculation. Results were multiplied by the total volume of the DNA preparation to provide the total copy number per insect, and were expressed as a *ftsZ*/18S ratio.

3. RESULTS

The digestion of *Z. pullula* genomic DNA with *Msp* I and *Hpa* II revealed that *Hpa* II left undigested several DNA fragments at high molecular weight in comparison with *Msp* I (figure 1*a*). *Msp* I and *Hpa* II are isoschizomers that recognize the same target sequence 5′-CCGG-3′, but while *Msp* I cleaves the target sequence irrespectively of the methylation status of the inner cytosine, *Hpa* II is inhibited by its methylation. The difference in the restriction pattern of the two isoschizomers indicated therefore that a portion of CpG doublets is methylated in the leafhopper. Furthermore, the lack of small products after *Msp* I digestion suggested that CpG sequences were not clustered in the *Z. pullula* genome (figure 1*a*). Finally, the absence of an extensive digestion with *Msp* I indicated

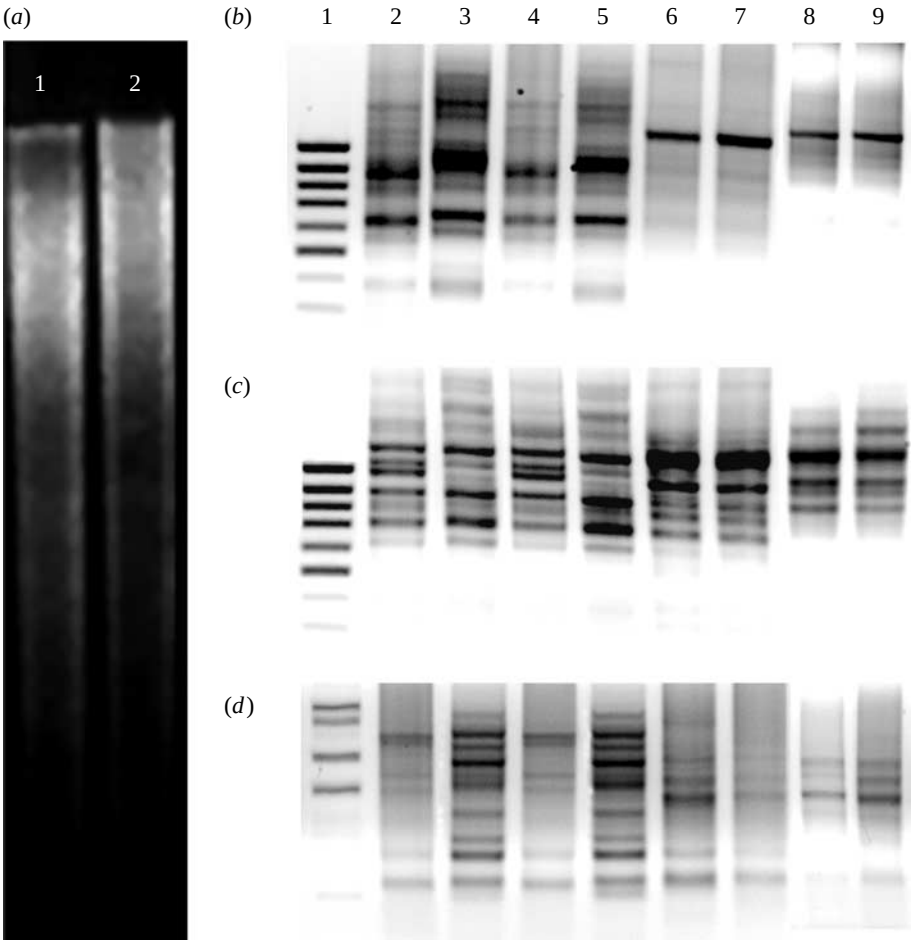

Figure 1. (*a*) The digestion of *Z. pullula* genomic DNA with *Msp* I (lane 1) and *Hpa* II (lane 2) reveals the presence of undigested DNA fragments after *Hpa* II, indicating the occurrence of genome methylation. (*b–d*) RAPD profiles obtained with DNA samples extracted by whole *Z. pullula* females (lanes 2–3), feminized males with ovaries (lanes 4–5), males (lanes 6–7) and feminized males with testes (lanes 8–9) after digestion with *Msp* I (lanes 2, 4, 6 and 8) and *Hpa* II (lanes 3, 5, 7 and 9), obtained with primers (*b*) OPA1, (*c*) RAPD 600 and (*d*) OPA4. Lane 1 (*b*) and (*c*), 100 bp ladder DNA marker; lane 1 (*d*), lambda/*Hind* III-digested DNA marker.

that the target sequence 5′-CCGG-3′ was not highly represented in the *Z. pullula* genome (figure 1*a*).

Methylation-sensitive RAPD PCR showed a very low level of variation in the profiles, comparing individuals of the same sex with each RAPD primer, indicating that results were reproducible. Variations in the RAPD results neither affected the number nor the size of the amplicons, but slightly the amount of DNA in some bands only. However, these differences never brought ambiguous results when sex-specific RAPD amplifications were compared.

Not all the RAPD primers were useful to detect sex-specific differences in the methylation patterns. With the primer OPA3, for instance, all the lanes showed the same results, indicating that the amplified fragments were not differently methylated in *Z. pullula* males and females (data not shown).

OPA1, OPA4 and RAPD 600 primers were useful for amplifying methylated regions, as evident if lanes digested with *Msp* I are compared with those digested with *Hpa* II (figure 1*b–d*). The primer OPA1 amplified two DNA fragments that were differently methylated, the first one is methylated in females and not in males, whereas the second one is methylated in males and not in females (figure 1*b*). On the contrary, primers RAPD 600 and OPA4 (figure 1*c*,*d*) evidenced a unique DNA band that is methylated in females and not in males.

Strikingly, all RAPD PCR profiles indicate that feminized males possess the same imprinting pattern of females (figure 1*b–d*).

The same experiments were performed on DNA extracted from testes and ovaries, confirming the occurrence of a sex-specific methylation of the genome, and strengthening the results obtained with somatic tissues, that is the presence in feminized male gonads of a female methylation pattern (data not shown).

In order to verify whether the female imprinting in feminized males was also related to the presence of a functional female gonad, a histological analysis of *Z. pullula* ovaries has been performed.

Gonad histological examination revealed that they possess either a typical ovariole histological architecture or an altered ovary's structure (figures 2 and 3). *Zyginidia pullula* ovaries are composed of telotrophic meroistic ovarioles, containing nurse cells and young oocytes located in a spindle-shaped germarium (figure 2*a*). The nurse cells show a basophilic cytoplasm with protrusions projected to a centrally located fibrous trophic core (figure 2*a–b*). Young oocytes growing into the vitellarium remain connected to the trophic core by nutritive cords (figure 2*d*). The pre-vitellogenic oocytes are characterized by a homogeneous cytoplasm filled with fine basophilic granules and a large transparent germinal vesicle

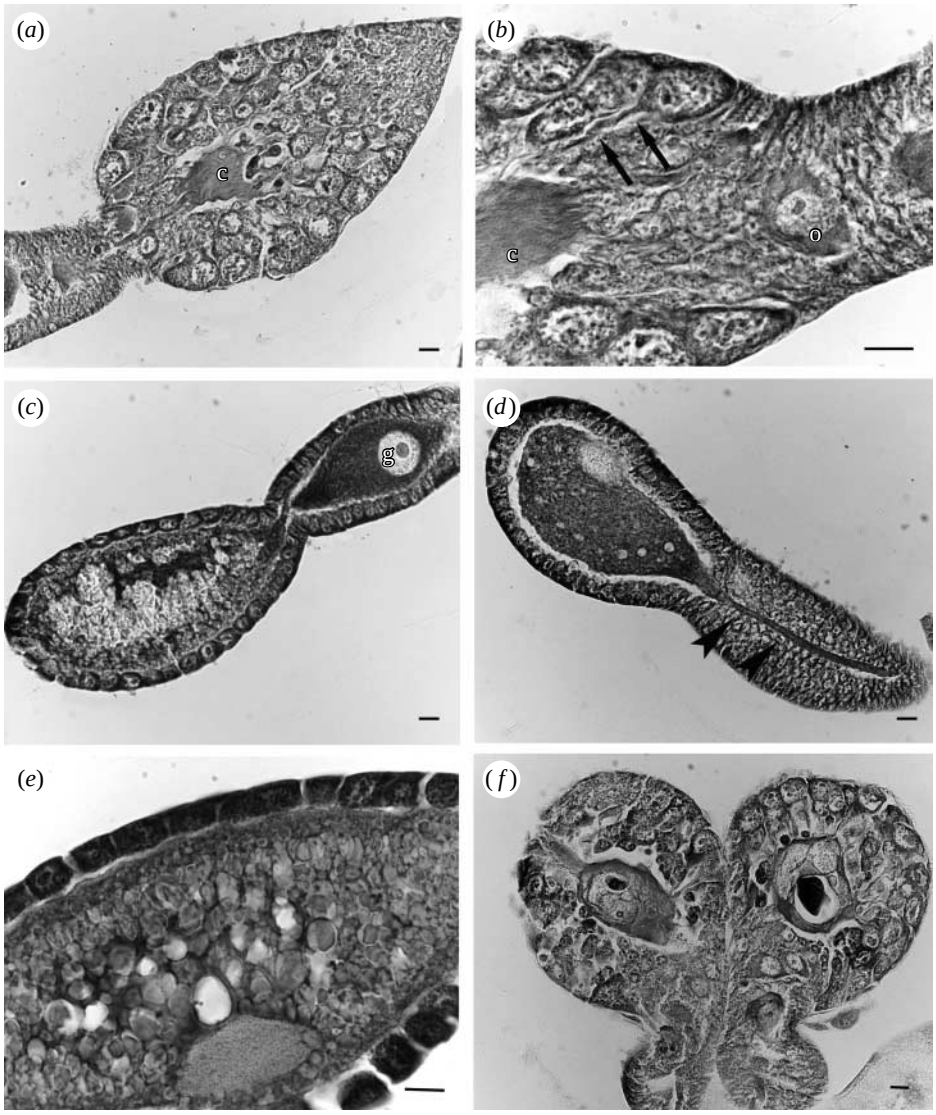

Figure 2. Ovary sections from feminized males stained with (*a–d*,*f*) gallocyanin–chrome alum reaction and (*e*) haematoxylin/eosin stain. (*a*,*b*) In some intersexes, the structural organization is similar to that of uninfected females with a spindle-shaped germarium containing basophilic nurse cells, with protrusions (arrow heads) connected to a central fibrous trophic core (c), and young oocytes (o). Different staged oocytes in the vitellarium region are shown in (*c–e*). (*f*) Two tightly adherent and disorganized germaria. Arrowheads, nutritive cords; g, germinal vesicle. Bar = 10 μm.

(figure 2*c–d*). However, in some feminized males, the ovarioles were found to be more adherent to each other (figure 2*f*) and characterized by an altered morphology of the germarium, which appeared reduced in size and irregularly shaped with no clearly polarized organization (figure 3*a*). A large number of nurse cells lost the syncytial architecture around the trophic core, decreased the adhesion with neighbouring cells and became round-shaped (figure 3*b*). The trophic core region appeared shrunken, with the fibrous structure hardly distinguishable and a high number of degenerating cells inside (figure 3*c*). In some ovarioles, cells with characteristic morphological alterations associated to apoptotic patterns were detected (figure 3*d*). Moreover, the pre-vitellogenic oocytes growing in the vitellarium showed a reduced and less compact cytoplasm; sometimes differently sized vacuoles containing heterogeneous granules were seen, and the ooplasm was also less adherent to the surrounding follicular epithelium (figure 3*e*). The yolky oocytes contained fewer and irregularly shaped granules (figure 3*f*). Some intersexes had the spermatheca full of sperm, indicating that they had successfully mated with males (data not shown). In addition to feminized males possessing ovaries, some very rare intersexes (less than 1% of all intersexes) surprisingly had male gonads. These specimens showed a wholly irregular anogenital zone, with the ovopositor composed of three pairs of valve that appeared malformed in structure compared with those of normal females (figure 4*a*) and bearing upper pygofer appendages (figure 4*b*). The histological analysis revealed gonads morphologically similar to those of normal males with single sac-like testes (figure 4*c*) containing differently staged germ cells and mature spermatozoa (figure 4*d*).

Remarkably, feminized males bearing testes showed the same methylation pattern of males (figure 1*b–d*) and, on average, *Wolbachia* density in these specimens was approximately four orders of magnitude lower than in infected females and intersexes with ovaries (*Wolbachia ftsZ* to *Z. pullula* 18S rDNA gene ratios: $4.9\times10^{-7}\pm3.1\times10^{-7}$, $7.1\times10^{-3}\pm4.6\times10^{-3}$ and $7.5\times10^{-3}\pm3.1\times10^{-3}$, respectively).

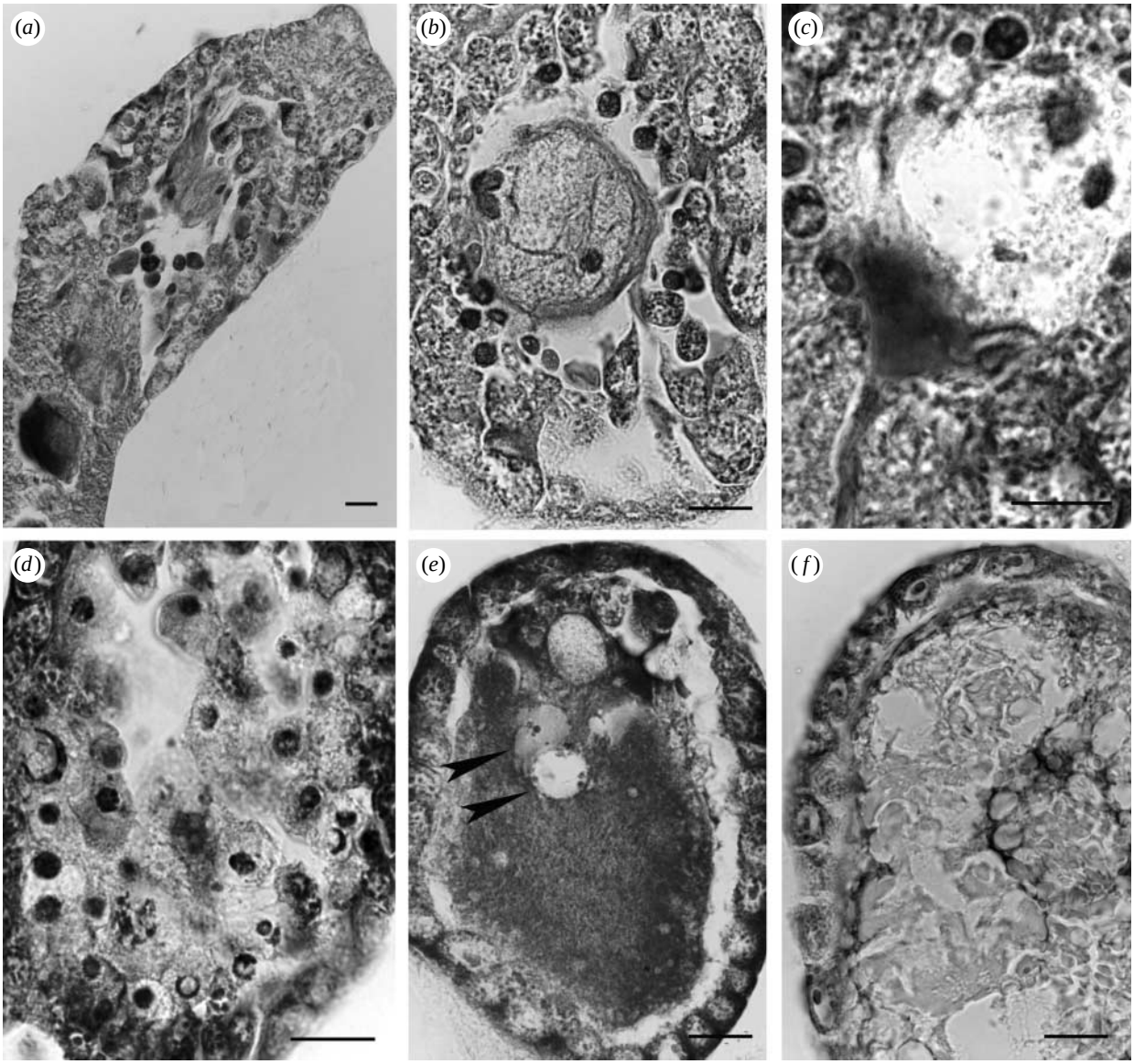

Figure 3. Ovariole sections from feminized males stained with gallocyanin–chrome alum reaction. (*a*) The germarium from some intersexes is reduced in size and irregularly shaped, (*a–c*) the adhesion among the adjacent round nurse cells and to the trophic core decreases. A particularly altered germarium structure with many cells showing apoptotic pictures is seen in (*d*). (*e*) Note a pre-vitellogenic oocyte with differently sized vacuola (arrow heads) in a cytoplasm, which reduces the adherence to the follicular epithelium, and (*f*) a yolky oocyte with loose and irregularly shaped granula. Bar = 10 μm.

4. DISCUSSION

A previous paper reported the feminization of *Z. pullula* genetic males by *Wolbachia*. Feminized males have morphological characteristics similar to those of typical females, with the exception of male features in the last abdominal segments (Negri *et al.* 2006).

In the present study, two kinds of *Z. pullula*-feminized males are found, bearing either ovaries or testes. Intersexes with testes are extremely rare (less than 1% of all intersexes). They possess a *Wolbachia* density four orders of magnitude lower than in infected females and intersexes with ovaries, as assessed by real-time PCR, suggesting a correlation between bacterial density and host phenotype. In this regard, even if qPCR analyses have been performed on adults and not on sexually undifferentiated individuals, we cannot exclude that the low *Wolbachia* titre would be responsible for the slight feminization degree, as observed in *E. hecabe* and in isopods (Rigaud & Juchault 1993; Narita *et al.* 2007). Indeed, male killing and cytoplasmic incompatibility induced by *Wolbachia* have been proved to be modulated by a bacterial cell density-dependent mechanism (Breeuwer & Werren 1993; Stouthamer *et al.* 1999).

Methylation-sensitive RAPD PCR allowed us to observe sex-specific differences in the methylation pattern of *Z. pullula*. Such differences in the two sexes are considered proof of correlation between DNA methylation and imprinting in both mammals and insects (Crouse 1960; Moore & Haig 1991; Bongiorni *et al.* 1999).

Interestingly, all RAPD PCR profiles show that *Z. pullula* feminized males with ovaries possess the same imprinting pattern as females. This indicates that *Wolbachia* infection disrupts male imprinting, which dramatically influences expression of genes involved in sex differentiation and development.

Feminized males display phenotypic features that are typical of females, including the production of eggs in place of sperm, and the ability to copulate with normal males (although progeny are only occasionally observed; Negri *et al.* 2006). This behaviour could be explained by

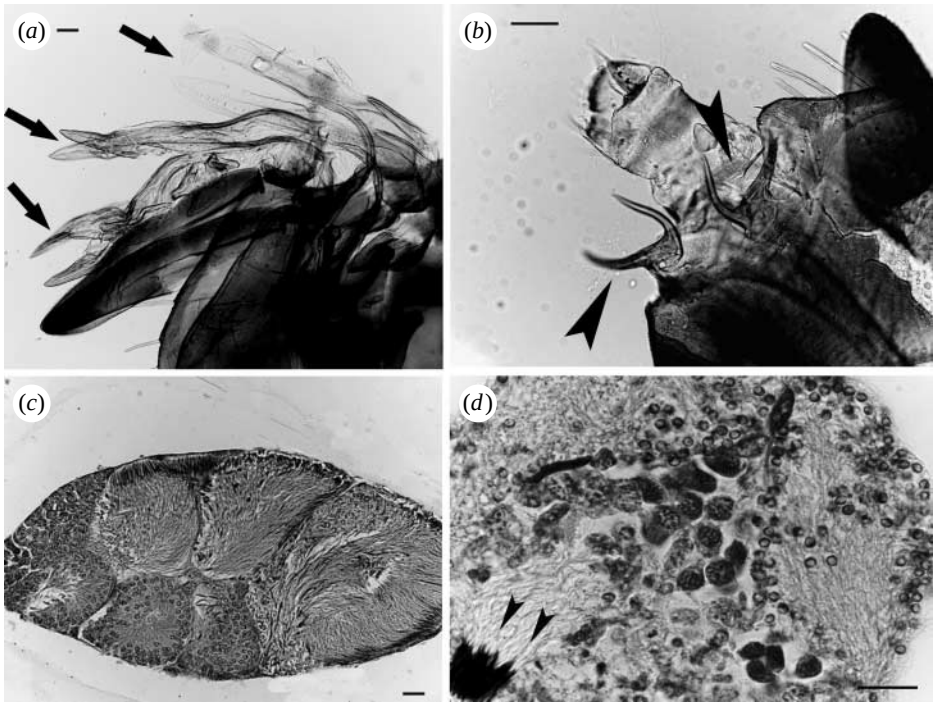

Figure 4. (*a*) Rare intersexes have a male gonad and an ovopositor with three pairs of malformed valvae (arrows), and (*b*) upper pygofer appendages, i.e. chitinous structures typical of males (arrowheads). The morphology of the testis is similar to that of normal males ((*c*) haematoxylin/eosin histological stain and (*d*) gallocyanin–chrome alum reaction). Arrowheads, mature spermatozoa, Bar = 50 μm (*a*,*b*) and 10 μm (*c*,*d*).

the morphological differences in ovarian structural organization that we observed in the feminized males versus true females. In the germarium, the nurse cells and trophic core appear particularly affected by the bacterial infection. Both nurse cells and oocytes are connected to the trophic core, which is rich in polarized and stable microtubules. These microtubules are directly involved in nurse cell–oocyte transport, via a cytoplasmic dynein-like motor, as demonstrated in *Rhodnius prolixus* (Harrison & Huebner 1997). The structural damage of nurse cells and trophic core we found in the germarium of some *Z. pullula* intersexes suggests a compromised ability to provide macromolecules and organelles for oocyte development. Indeed, oocytes growing in the vitellarium are affected, indicating an involvement of *Wolbachia* infection in gonad functioning, which causes a reduction in oocyte quality.

Feminized male leafhoppers are not completely female, since they still show male chitinous structures (Negri *et al.* 2006), thus it is clear that *Wolbachia* cannot completely establish female imprinting on the male genome. Furthermore, feminized males with testes show the same methylation pattern as males, suggesting that *Wolbachia* can alter the proper genomic imprinting only when it exceeds a threshold density.

Our data may support speculative hypotheses proposed on the basis of other *Wolbachia*/host interactions. In the wasp *Nasonia vitripennis*, the bacterium induces a segregation failure during the first mitotic division in unfertilized (haploid) eggs, producing nuclei that contain two identical sets of maternally derived chromosomes. Complete homozygosity at all loci would normally cause a wasp to develop as a sterile, diploid male, but these individuals are female. Since genomic imprinting seems to play a crucial role in the wasp's sex determination, *Wolbachia* should be able to induce not only a duplication of the whole genome, but also to imitate the paternal imprint (in one set of host chromosomes; Dobson & Tanouye 1998; Trent *et al.* 2006; Beukeboom *et al.* 2007). Even cytoplasmic incompatibility, probably the most widespread phenotype induced by *Wolbachia*, could be explained by a mechanism of epigenetic interference (Harris & Braig 2003). Paternal chromosomes are excluded from embryonic development in embryos affected by cytoplasmic incompatibility, and differential chromosome labelling, perhaps by DNA methylation, could be involved in the paternal chromosome loss.

Our data show that *Wolbachia* may disrupt proper genomic imprinting by altering the expression of several genes involved in sex determination and development simultaneously, thus avoiding the need for *Wolbachia* to interfere with each single gene separately.

Wolbachia, as an environmental factor experienced by a mother, can modulate epigenetic processes, altering her offspring's genomic imprinting. Thus, a new *Wolbachia* role as an environmental evolutionary force, inducing epigenetic trans-generational changes, should now be considered.

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