Wolbachia is not all about sex: male-feminizing Wolbachia alters the leafhopper Zyginidia pullula transcriptome in a mainly sex-independent manner

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Wolbachia causes the feminization of chromosomally male embryos in several species of crustaceans and insects, including the leafhopper Zyginidia pullula. In contrast to the relatively well-established ecological aspects of male feminization (e.g., sex ratio distortion and its consequences), the underlying molecular mechanisms remain understudied and unclear. We embarked on an exploratory study to investigate the extent and nature of Wolbachia’s effect on gene expression pattern in Z. pullula. We sequenced whole transcriptomes from Wolbachia-infected and uninfected adults. 18147 loci were assembled de novo, including homologs of several Drosophila sex determination genes. A number of transcripts were flagged as candidate Wolbachia sequences. Despite the resemblance of Wolbachia-infected chromosomal males to uninfected and infected chromosomal females in terms of sexual morphology and behavior, principal component analysis revealed that gene expression patterns did not follow these sexual phenotype categories. The principal components generated by differentially expressed genes specified a strong sex-independent Wolbachia effect, followed by a weaker Wolbachia-sexual karyotype interaction effect. Approaches to further examine the molecular mechanism of Wolbachia-host interactions have been suggested based on the presented findings.

Keywords: Wolbachia infection, male feminization, principal component analysis (PCA), Zyginidia pullula transcriptome, transcriptome de novo assembly, host-symbiont interactions

INTRODUCTION

Wolbachia is an intracellular symbiont alpha-proteobacterium that infects a wide range of arthropods and nematodes (Schulenburg et al., 2000; Werren et al., 2008). It is often transmitted vertically from females through the eggs to their future progeny; although, horizontal transfer between hosts has also been documented (Werren et al., 1995; Cordaux et al., 2001). Studying the mechanism of Wolbachia-host interactions is fascinating for many reasons. Wolbachia is capable of inducing several intriguing sex-related phenotypes in its hosts, including male killing (MK), in which infected males die during embryonic or larval stages; male feminization (MF), that is the development of genetic males into females; thylotokous parthenogenesis (TP) in which infected virgin females produce daughters. All of these phenotypes distort the progeny sex ratio in favor of females thus ensuring higher transmission rate of Wolbachia to the next generation of hosts (Werren et al., 2008; White et al., 2013). Another fascinating effect of the infection is cytoplasmic incompatibility between gametes (CI), which results in aberrant or considerably reduced offspring production, if uninfected females mated with infected males, or if the parents are infected with different Wolbachia strains (Werren et al., 2008; White et al., 2013). In this case, infected females possess a reproductive advantage compared to uninfected ones, and this again ensures the spreading of Wolbachia into the host population. Fast transition between the four phenotypes in the course of the coevolution of Wolbachia and its hosts hints that similar molecular mechanisms might underlie the apparently different effects (Ma et al., 2014). Due to its enormous host range, Wolbachia may have played a crucial role in the evolution of sex determination system and reproductive strategies in arthropods (Cordaux et al., 2011; Awrahman et al., 2014; Ma et al., 2014).

Various approaches have been employed to investigate the Wolbachia-host interactions in naturally infected and uninfected strains (Hoffmann et al., 1990; Negri et al., 2006; Riparbelli et al., 2012), experimentally inoculated cell lines (Noda et al., 2002; Xi et al., 2008), and antibiotic treated specimens (Hoffmann et al., 1990; Casiraghi et al., 2002). Although Wolbachia is an obligate intracellular symbiont naturally, protocols have been developed to keep it viable in cell-free media for days; however, no replication occurs in the extracellular phase (Rasgon et al., 2006; Gamston and Rasgon, 2007). The experimental/analytical techniques comprised a wide range including classical crossing and fecundity measurements (e.g., Hoffmann et al., 1990; Dunn et al., 2006), microscopic approaches (in situ hybridizations, electron microscope and immunohistochemical techniques for bacteria detection inside hosts and cells, tissues, etc.) (e.g., Negri et al., 2008; Fischer et al., 2011), gene expression analysis (e.g., Xi et al.,
Z. pullula (Negri et al., 2009a,b) and probably interacts with the insect hormone biosynthesis pathway to stimulate the production of feminizing hormones (Negri et al., 2010; Negri, 2012). In this study, whole transcriptomes of male and female Zyginidia samples (Wolbachia-infected and uninfected) were analyzed with Illumina deep sequencing technique, in order to understand the scope and nature of the Wolbachia-induced change in the host gene expression profile. Our initial idea was that if male feminization is the main consequence of Wolbachia infection, transcriptomes from the three female types (uninfected females, infected females and feminized males) should resemble each other and be different from the only phenotypically male group (uninfected males). In fact, we decided to test the hypothesis that sex reversal is Wolbachia’s main effect at the transcriptome level. Were this confirmed, we would proceed to identify differentially expressed genes between the two sexual phenotype groups.

METHODS

ZYGINDIA SPECIMENS

34 overwintering females of Z. pullula were collected in the same grass field in north Italy; and were reared individually in the laboratory as described in Negri et al. (2006). Overwintering females have often mated with several males (rarely with only one). By carefully examining the progeny, Wolbachia-infected (i.e., all female brood) and uninfected (i.e., male and female brood) lines were identified. Wolbachia infection was then confirmed by PCR on the mothers and randomly chosen samples from the brood as described in Negri et al. (2006). Morphological investigation as to the presence or absence of upper pygofer appendages lead us to separate feminized males from genetic females in the all-female (i.e., Wolbachia-infected) lines, and males and females in the uninfected lines. Males from uninfected lines were mated to the physiologically female progeny of the infected lines (consisting of genetic females and males) at each generation to produce the next generation of infected females (and feminized males). This backcrossing to uninfected males was done for at least three generations in the lab. Fifty adults from each of the four different categories of uninfected females (F), uninfected males (M), infected females (FW) and feminized (infected) males (MW) were pooled together for RNA sequencing.

cDNA LIBRARY PREPARATION AND SHORT-READ SEQUENCING

cDNA libraries were made from male and female specimens of infected and uninfected leafhopper lines. Infected males are phenotypically intersex and exhibit different degrees of feminization depending on the concentration of Wolbachia, ranging from individuals with functional ovaries to individuals with female secondary sexual characters, but possessing testes. We used thoroughly feminized infected males for RNA extraction. RNA purification, cDNA synthesis and Illumina library construction were performed using the protocols of Mortazavi et al. (2008), with the following modifications: total RNA, mRNA and DNA were quantified using a Qubit fluorometer (Invitrogen); mRNA fragmentation was performed using Fragmentation Reagent (Ambion) for a 3 min and 50 s incubation at 70°C and subsequently cleaned through an RNA cleanup kit (Zymo Research); additional DNA and gel purification steps were conducted using Clean and Concentrator kits (Zymo Research). Each sample library was sequenced as pair-ended 76-base reads on an Illumina Genome Analyzer II.
**DE NOVO TRANSCRIPTOME ASSEMBLY AND EXPRESSION LEVEL CALCULATION**

Due to the sensitive nature of *de novo* assembly, it is critical that the reads used to generate contigs have the highest sequencing quality. Reads were removed from consideration in the *de novo* assembly if they had a terminal *phred* (Ewing and Green, 1998) quality value less than 15, or contained more than 2 unknown nucleotides (i.e., N). Reads were also filtered due to similarities to known PCR primer and Illumina Adapter sequences. Using the reads pooled from all of the four samples that were not filtered out, the *de novo* assembly program Velvet (version 1.0.15) (Zerbino and Birney, 2008) was used in conjunction with a custom post-processing algorithm capable of retaining information from alternative splices (Sze et al., 2012) to assemble short reads into contigs, using sequence overlap information until the contigs could no longer be extended. Velvet was run under the following settings with a kmer length of 35: -cov_cutoff auto -max_branch_length 0 -max_divergence 0 -max_gap_count 0 -read_trkg yes. Sequenced reads that were kept as pairs and not filtered out together or separately were treated as “-shortPaired” with insert length of 175 bases and standard deviation of 75 bases. Single end reads that were not filtered out were treated as “-short.”

With the set of *de novo* assembled sequences serving as a reference, reads from each of the individual samples were mapped using the Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009). The number of reads that mapped to the contigs of each gene was tabulated and normalized to calculate FPKM (Fragments Per Kilobase Of Exon Per Million Fragments Mapped). Additional normalization among all samples was performed using the TMM protocol (Trimmed Mean of M-values) outlined in Robinson and Oshlack (2010), which takes into account differences in overall RNA populations across samples and is one of several methods used to evaluate RNA sequencing data. Normalization was implemented using the edgeR package in R (Robinson et al., 2010). All statistical analyses and graphs evaluating consistency between samples were produced using R v2.13.0 (R Development Core Team, 2011).

**GENE FUNCTIONAL ANNOTATION AND CLASSIFICATION**

Blast2GO v.2 (Götz et al., 2008) and WEGO (Ye et al., 2006) were used to obtain Gene Ontology (GO) annotations. Genes were also annotated using a BLASTX search (Altschul et al., 1990) (Expected value <1.00e-05) to the nr protein database available from GenBank as well as to the set of protein sequences available from the *Drosophila melanogaster* 5.34 and the pea aphid *Acyrthosiphon pisum* 2.1 releases. We chose the annotation with the highest BLAST score as long as the span of the alignment was greater than 80% of the length of the contig under query. For genes that did not report any hits, we lowered the minimum span to 40% of the length, choosing the annotation with the highest BLAST score having Expected value <1.00e-05.

**PRINCIPAL COMPONENT ANALYSIS OF GENE EXPRESSION VALUES**

Expression values were cleaned of extreme outliers, quartile-normalized and log-transformed before they were used for PCA. To make sure the result were not artifacts of the data preparation method, PCA was repeated on the raw (not normalized, not log-transformed) expression values as well as after several different outlier-filtering and normalization strategies. These statistical procedures were done in SAS 9.3.

**RESULTS**

**SHORT-READ SEQUENCING AND DE NOVO ASSEMBLY**

The mRNA population was analyzed with Illumina deep sequencing of male and female *Zyginidia* samples with and without *Wolbachia* infection. The pooled data from all samples had a total of 50 M pair-ended reads that were 76 bases long. All Illumina sequences are available for download at the NCBI Short Read Archive under the BioProject PRJNA171390. After sequences were filtered based on quality and matches to adapter and primer sequences, the 38 M reads from all four samples were pooled together and run through Velvet and the post-processing algorithm. Eventually, 18,147 loci and a total of 27,236 transcripts were assembled; multiple transcripts of a locus pertained often to different splicing isoforms and occasionally to largely differentiated alleles. The transcripts ranged in lengths from 291 bp to 15,389 bp, with mean and median lengths of 1006 bp and 702 bp, respectively. This assembly included a fairly large number of long transcripts: 25% were longer than 1250 bp and 10% were longer than 2000 bp. Of the 18,147 loci, 14,068 (77.5%) had a single isoform and the remaining 22.5% had multiple ones. Transcripts within a locus were subsequently collapsed into a single “representative locus sequence” by using ClustalW to run a multiple sequence alignment and identifying the locus consensus sequence. Mean and median lengths of consensus sequences were 900 bp and 618 bp, respectively. The total length of all loci consensus sequences was 16.3 Mb.

**GENE FUNCTIONAL ANNOTATION AND CLASSIFICATION**

6946 loci, corresponding to 38% of the entire dataset, were Gene Ontology annotated with Blast2GO. The consensus sequences were also aligned using a BLASTX search to the nr protein database available from GenBank as well as to the set of protein sequences available from Flybase and the aphid genome. Table 1 shows the proportion of cases that resulted in a hit where the length of the alignment was greater than 80% or 40% of the length of the query (leafhopper sequence). One might very crudely attribute the 80% alignment span hits to true genic homology and the 40% alignment span hits to conserved domains.

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**Table 1 | Summary statistics of *Zyginidia* transcripts homology search.**

|                      | 40% homology length | 80% homology length |
|----------------------|----------------------|----------------------|
| Genbank              | 60%                  | 39%                  |
| Flybase              | 48%                  | 26%                  |
| Pea aphid            | 81%                  | 32%                  |

40% homology length indicates that the length of the homologous segment covers at least 40% of the query (leafhopper) sequence. A correspondingly similar definition applies to the 80% homology length category. The percent values in the table cells show what percent out of all loci (18147) fit each criterion when Blasted against the designated dataset.
A number of genes potentially involved in the leafhopper sex determination were identified through homology search with the *Drosophila* sex determination genes. Although pea aphid is *Zyginidia*’s closest relative with a reference genome sequence (The International Aphid Genomics Consortium, 2010), the functional annotation for this genome is not as complete as that of *Drosophila*. Sex determining genes of pea aphid have been found based on homology with *Drosophila* sequences and lack direct experimental verification (The International Aphid Genomics Consortium, 2010). Therefore, we decided to use *Drosophila* sequences as the reference set. Figure 1 depicts the canonical sex determination pathway in *Drosophila*. Homologs of several *Drosophila* sex determination genes were identified among the transcripts including *dsx* (doublesex), *tra-2* (transformer-2), *vir* (virilizer), *fl(2)d* (female lethal d), *snf* (sans fille) and *ix* (intersex). No leafhopper homologs could be identified for *tra* (transformer), *sxl* (sex lethal), *fru* (fruitless) or *her* (hermaphrodite). Table 2 shows the expression levels for the identified leafhopper sex determination genes.

Seventeen genes in our dataset were flagged as likely *Wolbachia* sequences according to the Blast results against the NCBI dataset. Bacterial origin seems very probable for a number of these transcripts based on the expression levels in infected and uninfected lines, plus high similarity to known *Wolbachia* sequences (Table 3). These sequences were Blasted against the aphid genome to check if there was an indication of horizontal transfer; they were also Blasted against the *Drosophila* genome as a distant outgroup (Table 3).

**PRINCIPAL COMPONENT ANALYSIS OF TRANSCRIPTOMES**

Principal component analysis on transcriptomes of the four leafhopper samples surprisingly revealed that *Wolbachia* infection changes the host transcriptome extensively and the effect is by no means limited to sex-reversal. As evident in Figure 2,

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**Table 2 | Homologs of *Drosophila* sex determination genes in the *Zyginidia* transcriptome and their normalized expression levels.**

| Locus | Fly homolog | Multiple isoforms? | FW | MW | F | M |
|-------|-------------|-------------------|----|----|---|---|
| 5652  | dsx         | Y                 | 11.6 | 49.4 | 11.7 | 19.7 |
| 8743  | tra-2       | Y                 | 5.9  | 13.2 | 8.8  | 26.1 |
| 5015  | vir         | N                 | 20.2 | 9.9  | 16.1 | 23.2 |
| 10229 | fl(2)d      | N                 | 17.4 | 32.9 | 40.9 | 63.3 |
| 21060 | snf         | Y                 | 0    | 3.3  | 20.5 | 10.9 |
| 18743 | ix          | N                 | 5.9  | 11.6 | 81.8 | 43.9 |

FW, infected female; MW, infected feminized male (intersex female); F, uninfected female; M, uninfected male.
| Locus | Expression Genbank | Aphid (Acyrthosiphon pisum) homolog | Fly (Drosophila melanogaster) homolog |
|-------|-------------------|-----------------------------------|-----------------------------------|
|       | Accession         | F | M | Best hit | Accession | Q | Id | % E | Accession | Q | Id | % E |
| 1053  | 146.6             | 138.2 | 0 | 0.2 | GroEL [Wolbachia endosymbiont of Bemisia tabaci] | AFQ62607.1 | 62 | 93 | 5E-142 | ACYPI009253 | 57 | 37 | 2E-35 |
| 1097  | 51.8              | 37.9 | 1.5 | 0.6 | Outer surface protein precursor [Wolbachia endosymbiont of Bemisia tabaci] | ABF61215.1 | 88 | 83 | 8E-47 | - | - | - |
| 1331  | 60.4              | 39.2 | 0 | 0 | Molecular chaperone GroEL [Wolbachia endosymbiont of Bemisia tabaci] | WP 01040204.1 | 93 | 99 | 9E-75 | ACYPI009253 | 99 | 44 | 7E-24 |
| 13961 | 29.6              | 15 | 1.0 | 30S ribosomal protein S12 [Wolbachia endosymbiont of Cadra cautella] | WP 00329194.1 | 44 | 100 | 9E-7 | - | - | - |
| 23163 | 60.4              | 36.2 | 0 | 0 | Molecular chaperone GroEL [Wolbachia endosymbiont of Cadra cautella] | WP 007500179.1 | 53 | 58 | 2E-13 | ACYPI009253 | 98 | 43 | 1E-29 |
| 2673  | 93.3              | 40.9 | 51 | 0 | Ankyrin repeat domain protein [Wolbachia endosymbiont of Callosobruchus chinensis] | WP 006013682.1 | 98 | 68 | 8E-55 | - | - | - |
| 3433  | 93.3              | 21.3 | 17.4 | 1.0 | Ankyrin repeat domain protein [Wolbachia endosymbiont of Callosobruchus Chinensis] | WP 0006013682.1 | 98 | 68 | 8E-55 | - | - | - |
| 5403  | 51.8              | 32.9 | 0 | 0 | Ankyrin repeat domain protein [Wolbachia endosymbiont of Callosobruchus Chinensis] | WP 007550719.1 | 93 | 36 | 1E-13 | ACYPI009253 | 96 | 44 | 6E-14 |

(Continued)
the first PC (explaining 66.46% of variance) is highly correlated with all of the samples indicating that the expression of most genes is not significantly altered by Wolbachia and is similar across all samples. By the second PC (explaining 20.36% of variance), Wolbachia infected male and female samples cluster together and uninfected male and female cluster together. This PC is generated by genes whose expression is changed by Wolbachia consistently regardless of sexual karyotype or phenotype. The third PC (explaining 7.97% of variance) indicates an interaction term: F and M are similar and stand in the middle of the scale, with MW and FW occupying the opposite sides of them. This PC is generated by genes that are expressed similarly in uninfected males and females, and Wolbachia infection changes their expression in opposite ways in chromosomal males and females. Overall, sex inversion does not seem to be the only or even the biggest effect of Wolbachia on gene expression patterns in Zyginidia, even if it is the most conspicuous phenotypic consequence; otherwise, we would expect the three phenotypically female groups (F, FW, and MW) to cluster together and the only male group (M) to stand separate from them. None of the PCs show such a pattern.

PCA was repeated on expression values without the initial outlier filtering, and applying several different normalization and transformation strategies; they all yielded the same picture as described above: the main effect was invariably the presence or absence of Wolbachia regardless of sex (details not shown).

**DISCUSSION**

We assembled the Z. pullula transcriptome de novo and produced 18,147 loci and 27,236 transcripts with a total consensus sequence length of 16.3 Mb. These numbers were well within the expected range based on the aphid genome information. The aphid genome was reported to contain 11,089 highly supported RefSeq gene models with a total exonic length of 21.6 Mb; adding the gene models from six other gene prediction programs, a total of 34,604 non-redundant gene models with the total exonic length of 35.7 Mb were described (The International Aphid Genomics Consortium, 2010). The true number of genes is purportedly a number between those two estimates. Hence, our de novo assembly of the transcriptome seems to have captured a reasonable proportion of the expressed genes.

The results of sequence homology search (Table 1) confirm the closer relatedness of Z. pullula to A. pisum (the aphid) than to Drosophila. A caveat to this analysis is the extensive set of duplications in the aphid genome (The International Aphid Genomics Consortium, 2010). Without a leafhopper reference genome, we do not know if the same wave of duplications has affected Z. pullula or not; however, there was an indication in our data that it might have. By visual inspection of the sequences that were annotated as isoforms of a single locus computationally, we realized that some of them did not show signatures of known alternative splicing patterns; but looked like highly differentiated alleles (details not shown). These may indeed be paralogous sequences in the process of divergence. Further investigation, including the sequencing of single individuals rather than pools of them, will be required to separate paralogy from allelic variation.

A number of leafhopper sex determination genes were identified based on homology with fly sequences (Table 2).
Insect sex determination machinery has evolved around the transformer-doublesex axis (Sánchez, 2008); tra is the fast evolving component responsible for receiving the signal—sometimes through mediators—from the upstream sex determining factors (chromosomal constituent, incubation temperature, etc.), and dsx is the conserved switch that relays this signal down to the developmental processes (Sánchez, 2008; Verhulst et al., 2010). It is, therefore, not surprising that we found a homolog for dsx and not for tra in our dataset. The short length of the aligned segments prevented reliable assignment of male and female isoforms; but these initial results can be used to design primers to extract the whole genes from the leafhopper genome. Future experiments can then follow the flow of the signal in the sex determination pathway to identify where the cascade is diverted to female development in Wolbachia-infected genetic males. In the moth O. scapulalis, the impact point is somewhere above the level of dsx (Sugimoto and Ishikawa, 2012). Having the sequences of dsx male and female isoforms, one could check whether this is also true in leafhoppers. Unfortunately, the lack of replicates in our preliminary data makes it impossible to assess the significance of differential expression of genes across our four groups (FW, MW, F, and M). This is another task that remains to be done in future projects. In addition, development of X-linked sequence markers will enable early sexing of the embryos (based on the female XX/male XO karyotypes) through quantitative PCR; and facilitate the study of early developmental processes in infected and uninfected specimens.

We found a number of Wolbachia-related transcripts in the sequenced cDNA libraries (Table 3). The loci expressed mainly in infected lines with great similarity to known Wolbachia sequences are likely to have Wolbachia origin (e.g., loci 1053, 1097, 1331, and 13961). Curiously, a couple of loci are expressed primarily in the uninfected lines (e.g., locus 22635). At this point, we do not have a hypothesis as to the reason behind this observation. Repeating the experiments with replicates and higher sequencing depth would be the first step to confirm the reproducibility of these patterns. Our protocol of mRNA purification for creation of cDNA libraries involved a hybridization step with oligo-T ligands, which targets the eukaryotic mRNA poly-A tails; therefore, it will be necessary to employ a different purification strategy in order to capture most of the poly-A lacking bacterial mRNAs. Table 3 shows that several of the Wolbachia-related sequences code for Ankyrin-repeat proteins. Wolbachia genomes are well known for containing an extraordinarily high number of these genes (Wu et al., 2004; Iturbe-Ormaetxe et al., 2005). Gene transfer between Wolbachia and mosquito hosts has been previously reported (Woolfit et al., 2009). PCR experiments and phylogenetic analyses have confirmed horizontal gene transfer from bacterial endosymbionts to the aphid genome (The International Aphid Genomics Consortium, 2010). Similar approaches will be required to confirm bacterial or insect origin for the transcripts listed in Table 3. We tried to check for possible aphid lineage-specific horizontal transfers by asking whether a likely Wolbachia transcript shows high sequence similarity to an aphid sequence, but not a fly sequence; none of the loci in Table 3 expressed such a pattern. One of the Wolbachia-related transcripts showed a degree of homology with the aphid vasa gene (locus 4382). Almost identical homologs of this sequence exist in the three published Wolbachia genomes (Blast results not shown); its homologs in fly, leafhopper and the published Wolbachia genomes are characterized or predicted ATP-dependent RNA helicases. Vasa has been implicated in transmission of maternal effects and sex determination in clams (Milani et al., 2011). It will be very interesting to check if products
of host-homologous genes are actually exported out by Wolbachia into the host cell.

We used natural isolates of infected and uninfected leafhoppers for our comparisons with no antibiotic treatment. This relieved our comparisons from the confounding effects of antibiotic treatments on the host physiology. The rationale behind the traditional use of antibiotics to cure the infected lines from Wolbachia is to obtain infected and uninfected lines with the same genetic background. However, antibiotics can change the host physiology substantially, and quite remarkably, their effect can perpetuate through several generations of unexposed progeny (Ballard and Melvin, 2007; Zeh et al., 2012; Fridmann-Sirks et al., 2014). We avoided the use of antibiotics completely and achieved homogeneous genetic backgrounds among samples by taking advantage of repeated backcrossing of infected females to uninfected males. We collected all of our founder specimens from the same leafhopper population in a grass field. In the sampled population, the sex-ratio was only moderately female biased, with a moderate prevalence of the infection (~1:1.8 male:female, Wolbachia infection rate ~30% of the collected females; Negri I., unpublished data). As uninfected males are the only physiological males in existence, all the “egg-laying females” (in the field and in the lab, including the females used in this study) always mate with (and only with) uninfected males. Thus, all of our infected and uninfected lines come from the same genetic background. We carried out three further generations of backcrossing of infected females to uninfected males in the lab to effectively remove any residual genetic variation between the two groups. Details of rearing conditions are described in Negri et al. (2006). The natural pattern of sexual reproduction and the additional backcrossing done in the lab ensure the similarity of nuclear genetic backgrounds. We also tested mitochondrial gene sequences in Zyginidia samples from different Italian localities, both infected and uninfected, and they were all nearly identical (Negri I., unpublished data).

Through principal component analysis, we have showed that Wolbachia-induced changes in the host transcriptome are mainly sex-independent, and cannot be explained only by the sex reversal of genetic males. Previous transcriptomic studies on Wolbachia have reported changes in the expression of genes unrelated to the reproductive phenotype. For instance, Wolbachia infection in Armadillidium vulgare triggered the overexpression of immune-related genes (Chevalier et al., 2012). In the parasitoid wasp Asobara tabida, endosymbiont infection or lack thereof was associated with changes in expression of genes related to female reproductive development, iron and oxidative stress regulation, and immune recognition (Kremer et al., 2009, 2012). Artificial infection of Anopheles cell cultures by Wolbachia, surprisingly caused down-regulation of immune, stress response and detoxification genes (Hughes et al., 2011). Wolbachia-inoculated Drosophila cell lines exhibited differential expression of several GO categories not directly related to reproduction, including antimicrobial humoral response, ion homeostasis, response to unfolded protein and response to chemical stimulus (Xi et al., 2008). In Aedes aegypti, Wolbachia was shown to manipulate the expression of a metalloprotease gene through induction of a specific host miRNA (Hussain et al., 2011). Apart from such direct evidence, the observation of various forms of fitness cost in the feminized males, is consistent with the idea that sex reversal is not the sole effect of feminizing Wolbachia (Moreau et al., 2001; Rigaud and Moreau, 2004). Nevertheless, our study is the first one to quantitatively demonstrate that infection itself has a larger effect than that of sex reversion, through PCA of all of the available gene expression levels.

Lack of replicates meant that we could not quantitatively identify differentially expressed genes between the lines because we could not calculate variances. Instead, we focused on the global patterns of gene expression by applying PCA to gene expression values. Thousands of loci (each acting as one observation point) were used to generate the PCs. Antibiotic treatment and different genetic backgrounds could have been two potential sources of systematic bias in this type of analysis; they could have generated similar clustering patterns and confounded the interpretation of results. However, through the single-population sampling and the repeated backcrossing scheme, we avoided both sources of confusion.

Based on the PCA results, we encourage the use of biochemical bottom-up approaches focusing on the whole Wolbachia effect rather than the specific sex inversion event. Wolbachia’s effect is perceivably mediated by molecules secreted into the host cell or expressed on the outer membrane surface of the bacterium-containing vesicles. Wolbachia cannot be maintained in cell-free cultures indefinitely; but there are protocols to keep them alive in synthetic media for several hours (Rasgon et al., 2006; Gamston and Rasgon, 2007). In such a setting, the molecules released into the medium can be detected and purified using chromatographic and/or mass spectrometric approaches. Appropriate methods can be used, too, for isolation and characterization of surface molecules from the bacterium-containing vesicles. Pull-down experiments on the host proteins by these Wolbachia released or surface molecules might reveal the initial cellular targets of the endosymbiont-host interaction.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. J. Mol. Biol. 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Awrahman, Z. A., Champion de Crespigny, F., and Wedell, N. (2014). The impact of Wolbachia infection in Drosophila simulans on the evolution of host sex-determination mechanisms. Trends Genet. 27, 1–10. doi: 10.1016/j.tig.2011.05.002

Ballard, J. W. O., and Melvin, R. G. (2007). Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in Drosophila. Insect Mol. Biol. 16, 799–802. doi: 10.1111/j.1365-2583.2007.00760.x

Carasiri, M., McCall, J. W., Simoncini, L., Kramer, L. H., Sacchi, L., Genchi, C., et al. (2002). Tetracycline treatment and sex-ratio distortion: a role for Wolbachia in the moulding of filarial nematodes? Int. J. Parasitol. 32, 1457–1468. doi: 10.1016/S0020-7519(02)00185-3

Chevalier, F., Herbinière-Gaboreau, J., Charif, D., Mitta, G., Gavory, F., Wincker, P., et al. (2012). Feminizing Wolbachia: a transcriptomics approach with insights on the immune response genes in Armadillidium vulgare. BMC Microbiol. 12:51. doi: 10.1186/1471-2180-12-S1-S1

Corbula, R., Bouchon, D., and Grève, P. (2011). The impact of endosymbionts on the evolution of host sex-determination mechanisms. Trends Genet. 27, 332–341. doi: 10.1016/j.tig.2011.05.002

Corbula, R., Michel-Salzat, A., and Bouchon, D. (2001). Wolbachia infection in crustaceans: novel hosts and potential routes for horizontal transmission. J. Evol. Biol. 14, 237–243. doi: 10.1046/j.1420-9101.2001.00279.x

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with predicted alternative splices, single nucleotide polymorphisms and transcript expression estimates. Insect Mol. Biol. 21, 205–221. doi: 10.1111/j.1365-2833.2011.01127.x

Taylor, D. R. (1990). Evolutionary consequences of cytoplasmic sex ratio distorters. Evol. Ecol. 4, 235–248. doi: 10.1007/BF02214332

The International Aphid Genomics Consortium. (2010). Genome sequence of the pea aphid Acyrthosiphon pisum. PLoS Biol. 8:e1000313. doi: 10.1371/journal.pbio.1000313

Turelli, M. (1994). Evolution of incompatibility-inducing microbes and their hosts. Evolution 48, 1500–1513. doi: 10.2307/2410244

Verhulst, E. C., van de Zande, L., and Beukeboom, L. W. (2010). Insect sex determination: it all evolves around transformer. Curr. Opin. Genet. Dev. 20, 376–383. doi: 10.1016/j.gde.2010.05.001

Weeks, A. R., and Breeuwer, J. A. J. (2001). Wolbachia-induced parthenogenesis in a genus of phytophagous mites. Proc. R. Soc. B Biol. Sci. 268, 2245–2251. doi: 10.1098/rspb.2001.1797

Werren, J. H., Baldo, L., and Clark, M. E. (2008). Wolbachia: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6, 741–751. doi: 10.1038/nrmicro1969

Verhulst, E. C., van de Zande, L., and Beukeboom, L. W. (2010). Insect sex determination: it all evolves around transformer. Curr. Opin. Genet. Dev. 20, 376–383. doi: 10.1016/j.gde.2010.05.001

Zebnine, D. R., and Birney, E. (2008). Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18, 821–829. doi: 10.1101/gr.074492.107

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