Association of Wolbachia with Gene Expression in Drosophila Testes

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Abstract

Wolbachia is a genus of intracellular symbiotic bacteria that are widely distributed in arthropods and nematodes. These maternally inherited bacteria regulate host reproductive systems in various ways to facilitate their vertical transmission. Since the identification of Wolbachia in many insects, the relationship between Wolbachia and the host has attracted great interest. Numerous studies have indicated that Wolbachia modifies a variety of biological processes in the host. Previous studies in Drosophila melanogaster (D. melanogaster) have demonstrated that Wolbachia can affect spermatid differentiation, chromosome deposition, and sperm activity in the early stages of spermatogenesis, leading to sperm dysfunction. Here, we explored the putative effect of Wolbachia in sperm maturation using transcriptomic approaches to compare gene expression in Wolbachia-infected and Wolbachia-free D. melanogaster adult testes. Our findings show that Wolbachia affects many biological processes in D. melanogaster adult testes, and most of the differentially expressed genes involved in carbohydrate metabolism, lysosomal degradation, proteolysis, lipid metabolism, and immune response were upregulated in the presence of Wolbachia. In contrast, some genes that are putatively associated with cutin and wax biosynthesis and peroxisome pathways were downregulated. We did not find any differentially expressed genes that are predicted to be related to spermatogenesis in the datasets. This work provides additional information for understanding the Wolbachia-host intracellular relationships.

Keywords Wolbachia · Drosophila melanogaster · Transcriptomic · Intracellular relationship · Spermatogenesis

Introduction

Wolbachia, a genus of maternally transmitted intracellular bacteria, are widely distributed in arthropods and nematodes. To increase their chances of transmission, Wolbachia regulates the host’s reproductive system in various mechanisms, such as cytoplasmic incompatibility (CI), parthenogenesis induction (PI), male killing (MK), feminization, and enhancement of female fertility [1]. All these strategies are thought to provide a reproductive advantage to females infected with Wolbachia [1, 2]. Wolbachia are obligate mutualist and can significantly improve host fitness. For example, Wolbachia has been shown to be necessary for the growth and development of nematodes. When Wolbachia was removed from nematodes through antibiotic treatment, adult worms showed retarded growth, ovarian degeneration, and impaired embryo formation [3]. Wolbachia also can enhance the insect host’s resistance to many pathogens, such as RNA viruses [4, 5], and provide vitamins to the host [6]. Other processes, such as host longevity, olfactory responses, immunity, and stem cell proliferation, can be affected by Wolbachia [7–9]. These findings indicate that the relationship between Wolbachia and the host is complicated, and the related molecular mechanisms need to be explored.

Wolbachia infects a wide range of hosts and is distributed in various host tissues, suggesting that they interact with various host systems and pathways to ensure successful intracellular maintenance [10, 11]. Partridge’s group was the first to demonstrate that Wolbachia can activate insulin signaling and affect many metabolic pathways in the host [12]. Two studies in Drosophila cell lines using genome-wide RNAi screening and high-throughput fluorescence in situ hybridization technology showed that a great number of host genes might alter the Wolbachia titer, including genes involved in lipid
metabolism, transport, protein degradation, translation, and the cell cycle [13, 14]. These results indicate that, although some host biological processes have obvious impacts on the proliferation of Wolbachia, in this obligate mutualistic relationship, Wolbachia infection has little effect on components of the host’s innate immune pathways, such as Toll, IMD, JNK, and JAK-STAT. However, Wolbachia may impact some oxidative stress regulatory processes, such as reactive oxygen species (ROS) homeostasis, to ensure its maintenance in the host [15].

Over the past decade, RNA-seq has become an essential technique in transcriptomic studies, and Gutzwiller et al. used RNA-seq to study the dynamic expression of Wolbachia genes during the life cycle of Drosophila melanogaster (D. melanogaster), which provided a rich set of resources to further explore the functional basis of Wolbachia-Drosophila symbiosis [16]. He et al. analyze the ovaries of Wolbachia-infected and uninfected Drosophila adults and found that Wolbachia significantly affected many metabolic pathways, such as starch and sucrose metabolism, the TGF-β signaling pathway, galactose metabolism, the Wnt signaling pathway, and ubiquitin-mediated proteolysis [17]. RNA-seq is also used to study Wolbachia and host gene expression profile in other species, such as nematodes and spider mites [18–20]. The above results show that Wolbachia has multiple effects on their hosts.

In their insect hosts, Wolbachia is mainly located in components of the reproductive system, including the ovaries and testes [21], which are good tissues for studying the Wolbachia-host interactions. Male testes are the sites of spermatogenesis, which is a highly conserved process in Drosophila that is necessary for male reproduction [22]. In Drosophila adults, spermatogenesis begins in the apical region of the testis, where 6–12 germline stem cells (GSCs) lie in a rosette around a tight cluster of largely somatic support cells called the hub. GSCs asymmetrically divide to produce a new stem cell and gonialblast (Gb). Then, this Gb initiates four rounds of synchronous mitotic divisions with incomplete cytokinesis. Sixteen interconnected spermatogonia synchronously carry out a series of spermatocyte programs, such as DNA replication, cell growth, meiosis, and transcription of terminal differentiation genes. Subsequently, these 16 spermatocytes produce 64 mature spermatozoa [23].

The process of spermatogenesis in D. melanogaster differs at different developmental stages. Testes in Drosophila larvae and early pupae contain several groups of developing primary spermatocytes, but no cell from later developmental processes [24]. Gene expression also varies in the different stages of spermatogenesis. Studies have shown that cell cycle–related genes are significantly enriched at the top of the testis, where the mitotically dividing spermatocytes are located, while genes related to the cytoskeleton, nucleus, mitochondria, and ubiquitin are significantly enriched at the base of the testis. These genes contribute to the formation of mature sperm [25]. Wolbachia is present in GSCs from the beginning of spermatogenesis, although the density is extremely low (4–5 Wolbachia per cell). During the transformation from Gb to spermatogonia, the 16 interconnected spermatogonia cells will have unevenly partitioned Wolbachia. Then, from spermatogonia to spermatocyte, many spermatocytes appear completely uninfected. When spermatocytes enter the meiotic stage, Wolbachia is closely associated with microtubules, which aids in their entry into offspring cells. In the late stages of spermatogenesis, sperm cells undergo a series of changes, and Wolbachia is removed from the cells during the final stage of sperm maturation [15].

Some studies have shown that Wolbachia affects processes in early spermatogenesis, such as male meiosis, sperm motility, chromatin condensation [26], and spermatid differentiation [27]. However, in Drosophila, mature sperm cells are continuously produced in adults but not in the larval stage [23], and we believe that the impact of Wolbachia on the host’s reproductive organs may be different in the adult and larval stages. Therefore, this study aimed to explore the putative effects of Wolbachia strain wMel on the process of sperm maturation, based on an analysis of gene expression in the testes of D. melanogaster adults with and without Wolbachia infection. Wolbachia strain wMel has been widely used in the study of Wolbachia-host interactions [13, 28–30]. WMel is not necessary for the survival of Drosophila hosts, but it can improve the fitness of the hosts in many ways. Therefore, we selected Wolbachia wMel–infected and uninfected D. melanogaster adult testes for RNA-seq and analyzed the functions of the differentially expressed genes. Our research provides a new resource for studying the dynamic interactions of Wolbachia with their hosts.

### Methods

#### Fruit Fly Rearing

Drosophila melanogaster is naturally infected with Wolbachia wMel, hereafter referred to as DmelW, which were kindly donated by Prof. Hu Haoyuan (Anhui Normal University). Five bacterial genes (hcpA, fhpA, coxA, fisZ, and garB) were amplified by PCR and sequenced. These sequences were submitted to the MLST website for Wolbachia strain identification, and we found that D. melanogaster was indeed infected with Wolbachia strain wMel (Additional File 1). Uninfected Wolbachia line derived from the DmelW line, hereafter referred to as DmelT, was generated with tetracycline treatment. The DmelT strain was transferred to a standard medium and continuously cultivated for more than 10 generations to eliminate the effects of antibiotics; the feed was based on a standard Drosophila corn flour medium. The
fruit flies were reared in an artificial climate box (Ningbo Jiangnan Instrument Factory, China), under a 14:10-h light:dark cycle, with 7000 lx of light and 40% relative humidity [31] and under non-crowded conditions (100 ± 10 flies per 100-mL vial of the medium in 300-mL disposable plastic cup). Pontier et al. have reported that the communication between *Drosophila* pupae and the existence of *Wolbachia* in pupal stage could affect gamete compatibility [32]. We speculate that *Wolbachia* may have some unknown effect on communication between pupae of different sexes. In this consideration, we collect the DmelW and DmelT pupae after the pupation and keep the pupae apart from each other. Then, we collect male and female adults after their emergence, and raised separately in new cups with medium under the same conditions to rule out the possible effects of sexual perception on the physiological function of *D. melanogaster* [33].

**RNA Sequencing**

Male 1-day-old DmelW and DmelT fruit flies were dissected in RNase-free water, and the complete testes were placed in RNAAhold and stored at −80 °C until use. In this study, the testes of approximately 10 independent virgin males were pooled as a sample, and three biological replicates were carried out for both DmelW and DmelT. Total RNA was extracted from each sample using the TransZol Up Plus RNA Kit (TransGen, Beijing, China) and sequenced by the BGISEQ-500 platform (BGI, Shenzhen, China) to obtain PE150 bp paired-end transcriptome data in the size of 6 Gb. The data were submitted to the NCBI under accession number PRJNA639180.

**Differential Gene Expression Calculations and Enrichment Analyses**

We used Bowtie 2 v2.2.5 for genomic reference alignment of the clean sequencing reads and RSEM to calculate the gene expression levels of each sample [34]. DESeq software was used to detect differentially expressed genes according to the method described by Wang [35]. Differentially expressed genes (DEGs) were selected based on a fold change ≥2 and a p value < 0.001, with three biological replicates. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the DEGs was performed using KOBAS [36]. Gene ontology (GO) enrichment analysis of the DEGs was performed using the clusterProfiler R package. We convert the gene symbol of DEGs into ENTREZID based on the bioconductor annotation data (org.Dm.eg.db, [https://bioconductor.org/help/search/index.html?q=org.Dm.eg.db](https://bioconductor.org/help/search/index.html?q=org.Dm.eg.db)), and then using the enrichGO with default parameters to find the significant enriched GO terms and GO terms were considered enriched when p value < 0.05 and q value < 0.5 was obtained [37].

**Quantitative Real-time PCR (qRT-PCR) (Table 1)**

To further investigate the DEGs identified by RNA-Seq, 26 DEGs were selected for validation by qRT-PCR analysis. Specific primers for the 26 genes and *RpL32* (*D. melanogaster* ribosomal protein L32, as a reference gene) were designed using NCBI primer-BLAST (listed in Table S1). We selected the genes for qRT-PCR that not only the Log2 value (DmelW/DmelT) in transcriptome data was high but also the level of expression in each sample was high; for some genes, although the Log2 value (DmelW/DmelT) was very high, if the expression level was very low, we did not select them. RNA was extracted from 10 independent virgin males of DmelW and DmelT flies for each sample using the TransZol Up Plus RNA Kit (TransGen, Beijing, China), according to the manufacturer’s instructions. RNA reverse transcription was performed with 1 μg of the extracted RNA using TranscriptOne-Step gDNA Remover and cDNA Synthesis SuperMix (TransGen, Beijing, China), by adding 1 μL of gDNA remover, 1 μL of oligo(dT), 10 μL of 2× TS Reaction Mix, 1 μL of TransScript RT/RI Enzyme Mix, and nuclease-free water for a total volume of 20 μL. The reaction conditions were 42 °C for 30 min, followed by 85 °C for 5 s. To verify the DEGs, we used the PerfectStart Green qPCR SuperMix Kit (TransGen, Beijing, China). Each reaction contained 1 μL of cDNA, 0.4 μL each forward and reverse primer (10 μM/L), 10 μL of 2× PerfectStart Green qPCR SuperMix, and 8.2 μL of nuclease-free water. qRT-PCR was performed on Step One Plus qRT-PCR System (ABI). The reaction conditions were as follows: pre-reaction at 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s and 60 °C for 30 s, and a final dissolution step. The relative expression of each gene, comparing DmelW to DmelT, was normalized against the reference gene (*RpL32*) using the 2^−ΔΔCT method.

**Data Analysis**

For the data analysis, a multiple *t* test was performed in GraphPad Prism 8 to analyze the significance of differences between both groups. A *p* value assessed by the Holm-Sidak method and less than 0.05 was considered significant.

**Results**

**Transcriptome Sequencing Data from Adult Drosophila Testes**

We performed transcriptome sequencing of the testis tissues from DmelW and DmelT flies. A total of 6 samples were tested using the BGISEQ platform, with an average yield of 45.28 million clean reads per sample. The average alignment ratio of the sample comparison genome was 90.51%. The
average Q30 of each sample was 89.63. The data quality of all samples is very high and similar. Comparison of the gene expression profiles revealed 472 significant DEGs (with at least a twofold change, \(q\) value < 0.1%); 403 genes were upregulated, and 69 genes were downregulated (Table S2) in the presence of Wolbachia. DEGs account for 3.48% of the total detected genes.

### The DEGs in Testes from DmelW and DmelT Are Involved in Many Biological Processes

We performed gene ontology classification analysis of the 472 significant DEGs. Among the upregulated genes, the top 15 biological process (BP) classes were mostly involved in metabolism and transport processes, while most cellular component (CC) classes were involved in the vacuole and nuclear chromatin. Most molecular function (MF) classes were involved in hydrolase activity, including serine-type peptidase and mannosidase activity. Significantly downregulated genes were involved in wax biosynthetic and fatty-acyl-CoA metabolic process with fatty-acyl-CoA reductase (alcohol-forming) activity (Fig. 1; Table S3). The results of the KEGG enrichment analysis showed that the upregulated genes in the DmelW testes were mostly involved in other glycan degradation, lysosome, neuroactive ligand-receptor interaction, immune response, carbohydrate metabolism, and lipid metabolism, while the downregulated genes were involved in the peroxisome pathway (Fig. 2; Table S4). The main pathways associated with the DEGs between DmelW and DmelT testes are presented below.

### Carbohydrate Metabolism

Based on the KEGG enrichment analysis, multiple genes related to carbohydrate metabolism, especially sucrose and starch hydrolysis to produce \(\alpha\)-glucose, including Mal-A1, Mal-A2, Mal-A3, and Mal-A4, were significantly upregulated in DmelW testes (Fig. 3a). Among them, Mal-A1 was upregulated nearly 700-fold, and Mal-A2 was upregulated more than 100-fold; Mal-A3 and Mal-A4 were also upregulated fivefold and 15-fold in the DmelW testes (Fig. 3a).

### Lysosome and Neuroactive Ligand-Receptor Interactions

Several genes related to the lysosome and neuroactive ligand-receptor interaction pathways were upregulated in DmelW testes (Table 2). Seven mannosidase genes (LManI, LManII, LManIII, LManIV, LManV, LManVI, and Gba1a), a cell surface receptor signaling pathway gene (Tsp29fa), two aspartic type protease genes (CG31928 and CG31926), two sterol transporter genes (Npc2e and Npc1b), and a proton

| Table 1  | DEGs selected for qRT-PCR validation |
|----------|--------------------------------------|
| Relative expression | Gene symbol | log2 (DmelW/DmelT) | Biological functions |
| Upregulated | mag | 5.84 | Negative regulation of juvenile hormone biosynthetic process; lipase activity |
| | CG10116 | 6.63 | Lipid catabolic process |
| | Acox57D-d | 1.66 | Lipid catabolic process |
| | rdgA | 1.53 | Cellular lipid metabolic process |
| | Mal-A1 | 5.83 | Carbohydrate metabolic process |
| | Mal-A2 | 3.64 | Carbohydrate metabolic process |
| | Mal-A3 | 9.78 | Carbohydrate metabolic process |
| | Mal-A4 | 7.13 | Carbohydrate metabolic process |
| | Drs | 1.11 | Antimicrobial humoral response |
| | DrsI3 | 1.88 | Defense response to fungus |
| | Takl | 2.02 | Protein phosphorylation |
| | DptB | 1.41 | Response to bacterium |
| | spheroide | 8.48 | Defense response to gram-positive bacterium |
| | PGRP-SC2 | 1.54 | Humoral immune response |
| | CG8834 | 3.08 | Lipid biosynthetic process |
| | Npc2d | 4.33 | Intracellular cholesterol transport |
| | Npc2f | 1.34 | Intracellular cholesterol transport |
| | LManII | 1.76 | Mannose metabolic process; protein deglycosylation |
| | LManVI | 4.14 | Mannose metabolic process; protein deglycosylation |
| | Vha100-5 | 1.08 | Proton transmembrane transport |
| Downregulated | CG10097 | −1.30 | Wax biosynthetic process |
transmembrane transporter gene (vha100-5) were enriched in the lysosome pathway. Among these genes, LManII and LManVI were upregulated nearly 1.5-fold, and vha100-5 was upregulated twofold by qRT-PCR (Fig. 3b). Seven serine protease genes enriched in the neuroactive ligand-receptor interaction pathway, including betaTry, alphaTry, zetaTry, iotaTry, and etaTry, were also upregulated in DmelW testes. Molecular function analysis showed that all seven genes were involved in serine-type peptidase activity (Table S4).

**Lipid Metabolism**

GO biological process analysis showed that many genes were involved in lipid biosynthetic processes, including CG11453, AstA, AstC, CG5568, rdgA, Pxt, CG9993, jhamt, CG17999, CG11162, CG8834, CG15533, and CG15534 (Table 3). Among these lipid biosynthesis genes, jhamt (juvenile hormone acid methyltransferase) participates in the biosynthesis of juvenile hormone III, and rdgA is associated with phosphatidyl biosynthesis. GO analysis showed that the genes CG11453, CG5568, Pxt, CG9993, CG17999, and CG8834 are involved in fatty acid biosynthesis, and CG11162 is involved in steroid biosynthesis. Finally, several genes involved in steroid transport were significantly upregulated in the testes of Wolbachia-infected D. melanogaster. Among these genes, Npc2f and Npc2d are involved in intracellular cholesterol transport (Fig. 4a). We also found 11 genes involved in lipase activity, including mag and CG10116, that were significantly upregulated in DmelW testes (Table 3; Fig. 4a).

**Immune Responses**

We detected six upregulated genes in DmelW testes involved in the innate immune pathway of Drosophila that function in the Toll and IMD pathways, including Drs, DptB, spheroid, Tak1, and Drs13, as well as PGRP-SC2, which encodes a peptidoglycan recognition protein (Fig. 3c; Table 2).
Peroxisome Pathway

Among the downregulated genes in DmelW, most of the genes in the BP GO class were involved in the wax biosynthetic process. KEGG pathways related to the peroxisome pathway were enriched, including several peroxisome membrane protein-encoding genes: CG10097, CG1441, CG17560, and CG13091 (Table 3). The downregulation of CG10097 was verified by qRT-PCR (Fig. 4b). BP analysis showed that these genes were involved in the wax biosynthetic process with fatty-acyl-CoA reductase (alcohol-forming) activity. We further examined the expression of several genes related to reactive oxygen species (ROS) degradation, including catalase (cat), peroxidase (pxd), superoxide dismutase 1 (sod1), and two glutathione-S-transferase-encoding genes (GstE10 and GstE9) in DmelW and DmelT by qRT-PCR, and only detected slight downregulation of pxd in DmelW (Fig. 3d).

Discussion

The interaction between Wolbachia and its host is dynamic and complex and has yet to be fully clarified [38]. Wolbachia are mainly located in the reproductive system of their insect hosts [21], including the female ovaries and male testes, which makes them good tissues for studying Wolbachia-host interactions. In recent years, the interactions between Wolbachia and its hosts have been widely studied. Most studies have focused on the mechanisms of CI induced by Wolbachia. Zheng et al. showed that Wolbachia induced high expression of key gene in adult D. melanogaster testis, but low expression levels in female ovaries infected with Wolbachia. Overexpression of key in the testes significantly reduced the embryo hatching rate, and Wolbachia-infected females could rescue this defect. Overexpression of key also altered the expression of some immunity-related genes and increased ROS levels in male testes. These results suggest that Wolbachia may induce fertility defects through immune-related pathways [39]. This is similar to the results of the study on Hira gene by Zheng et al. [40]. Zheng et al. performed an analysis of small RNAs in adult D. melanogaster testes infected with Wolbachia and uninfected testis and showed that Wolbachia may negatively regulate psq by upregulating nov-miR-12, resulting in male fertility disorders [41]. Yuan et al. conducted a proteome analysis of female Drosophila spermathecae and seminal receptacles (SSR) and showed that Wolbachia infection significantly altered the expression of various proteins in
males, including immune-, metabolic-, and reproductive-related proteins. *Wolbachia* infection leads to downregulated expression of male reproductive-related proteins [42]. Compared with the findings of Zheng et al. and other transcriptome data from *Drosophila* testes, our transcriptome data from the testes of *Wolbachia*-infected and uninfected male *Drosophila* are different. Our transcriptome data did not show differential expression of reproductive-related genes in DmelW and DmelT. In contrast, we found that carbohydrate metabolism, proteolysis, and immune-related genes were greatly upregulated in DmelW, which was consistent with the results of Zheng et al. [26]. In particular, in our transcriptome data, the expression of genes key, *Hira*, and *Ance*, which were reported to be related to the reproduction of male *D. melanogaster* and may be the cause of *Wolbachia*-induced CI [27, 39], was not significantly different between *Wolbachia*-infected and uninfected male testes. Zheng et al. studied the effects of *Wolbachia* on spermatogenesis in the larval stages of *Drosophila* [26], and we believe that the effects of *Wolbachia* on spermatogenesis in the larval and adult stages are different, especially as not all sperms contain *Wolbachia* in the early stages of spermatogenesis, but almost 100% can induce CI [43]. *Wolbachia* is thought to affect spermatogenesis by secreting its own substances. The dynamic changes in *Wolbachia* gene expression in the different life cycles of *D. melanogaster*, identified by Darby et al., showed that the genes related to the *Wolbachia* secretory system were upregulated in the larval and pupal stages. During this period, *Wolbachia* may secrete more proteins that participate in *Wolbachia*-host interactions [18]. Based on the results above, we believe that *Wolbachia* may have different interactions with the host during the larval and adult stages. *Wolbachia* may have a more significant effect on male testes in the early stage of spermatogenesis through protein secretion, and this effect may be weaker in the adult stage.

**Wolbachia Infection Is Associated with More Active Carbohydrate Metabolism in the Host**

Pyruvate is the most essential metabolic molecule in prokaryotes, and it is also necessary for *Wolbachia* survival. Pyruvate is produced by glycolysis, and the *Wolbachia* genome lacks three key enzymes to produce pyruvate. However, *Wolbachia* retains the complete pyruvate pathway to generate energy through the tricarboxylic acid (TCA) cycle [44, 45]. Glycolysis is accelerated in *Wolbachia*-infected nematodes and that nematodes provide pyruvate for their symbiotic bacteria [44]. In our study, we noticed that carbohydrate metabolism was more active in *Wolbachia*-infected samples. The expression levels of the Mal-A2, Mal-A1, Mal-A3, and Mal-
A4 genes, which are involved in starch and sucrose metabolism, were significantly upregulated in DmelW testes. The hydrolytic enzymes encoded by these genes can accelerate D-glucose formation, which is the initial substrate of glycolysis [46]. This is similar to the results reported by Zheng et al. [26]. Therefore, Wolbachia may compete with the host to consume the glycolysis substrate glucose, resulting in accelerated production of glucose in the host.

**Wolbachia May Rely on the Host Lysosome Pathway for Amino Acids**

Due to its intracellular lifestyle, Wolbachia lacks many essential biosynthetic pathways, many of which are involved in amino acid production [47, 48]. Therefore, Wolbachia must obtain amino acids from their hosts [47–50]. Previous studies have shown that Wolbachia is highly dependent on host proteolysis via ubiquitination and the endoplasmic reticulum-associated protein degradation (ERAD) pathway [14]. Yuan et al. also speculated that Wolbachia might alter the abundance of proteins in the SSR by affecting ubiquitin-proteasome-mediated proteolysis [42]. Although there is no significant difference in ubiquitin-related genes between DmelW and DmelT, we found significant differences in lysosome-related genes between the two strains, indicating that lysosome activity was significantly enhanced in Wolbachia-infected testes and that lysosomes play an important role in the process of

| Main significantly enriched KEGG pathways associated with upregulated DEGs in DmelW testes | log2 (DmelW/DmelT) | q value | KEGG pathway | Biological functions |
|---|---|---|---|---|
| Carbohydrate metabolism | Mal-A1 | 5.83 | 4.38E−133 | Starch and sucrose; galactose metabolism | Carbohydrate metabolic process |
| | Mal-A2 | 3.64 | 2.53E−106 | Starch and sucrose; galactose metabolism | Carbohydrate metabolic process |
| | Mal-A3 | 9.78 | 2.41E−146 | Starch and sucrose; galactose metabolism | Carbohydrate metabolic process |
| | Mal-A4 | 7.13 | 5.18E−156 | Starch and sucrose; galactose metabolism | Carbohydrate metabolic process |
| Lysosome | LManIII | 2.51 | 1.17E−100 | Lysosome | Mannose metabolic process; protein deglycosylation |
| | LManI | 1.76 | 1.03E−85 | Lysosome | Mannose metabolic process; protein deglycosylation |
| | LManII | 1.08 | 1.21E−272 | Lysosome | Mannose metabolic process; protein deglycosylation |
| | LManVI | 4.14 | 0 | Lysosome | Mannose metabolic process; protein deglycosylation |
| | LManV | 5.94 | 9.50E−272 | Lysosome | Mannose metabolic process; protein deglycosylation |
| | LManIV | 2.91 | 1.53E−4 | Lysosome | Mannose metabolic process; protein deglycosylation |
| | Gba1a | 1.86 | 1.76E−33 | Lysosome | Membrane lipid catabolic process |
| | Tsp29Fa | 2.11 | 3.03E−52 | Lysosome | Cell surface receptor signaling pathway |
| | Vha100-5 | 1.08 | 2.05E−31 | Lysosome | Proton transmembrane transport |
| | CG31928 | 1.270291563 | 3.39E−4 | Lysosome | Proteolysis |
| | CG31926 | 1.889485699 | 8.48E−4 | Lysosome | Proteolysis |
| | Npc2e | 5.889485699 | 7.41E−08 | Lysosome | Organic hydroxy compound, sterol transport |
| | Npc1b | 4.040493487 | 5.44E−35 | Lysosome | Organic hydroxy compound, sterol transport |
| Immune pathway | DptB | 1.41 | 7.49E−10 | Toll and IMD signal pathway | Response to bacterium |
| | Spheroid | 8.48 | 1.01E−70 | Toll and IMD signal pathway | Defense response to gram-positive bacteria |
| | Drs | 1.11 | 2.97E−22 | Toll and IMD signal pathway | Antimicrobial humoral response |
| | Takl1 | 2.02 | 1.22E−16 | Toll and IMD signal pathway | Protein phosphorylation |
| | PGRP-SC2 | 1.54 | 2.11E−7 | Toll and IMD signal pathway | Humoral immune response |
| | Drsl3 | 1.88 | 1.85E−04 | Toll and IMD signal pathway | Defense response to fungus |
intracellular protein degradation [51]. Thus, we speculate that amino acids may be acquired by Wolbachia via the lysosomal degradation pathway of the host. This is consistent with the results of previous studies in Tetranychus urticae [52]. Besides, several serine protease-encoding genes were upregulated in DmelW, including betaTry, alphaTry, zetaTry, iotaTry, and etaTry. KEGG enrichment analysis showed that the neuroactive ligand-receptor interaction pathway was significantly enriched in Wolbachia-infected testes. Considering these results, we speculate that Wolbachia can increase host catabolism. Similar results were reported by Zheng et al. [26].

| Relative expression | Genes                                                                 | Biological functions               | KEGG pathway |
|---------------------|----------------------------------------------------------------------|-----------------------------------|--------------|
| Upregulated         | CG18258, Yp1, Yp2, CG6296CG3699, CG6277, CG10116, CG15533, CG15534   | Lipid catabolic process           | NA           |
|                     | Npc1b, Npc2f, Npc2e, Npc2d mag                                      | Sterol transport; intracellular cholesterol transport | NA           |
|                     | CG11162                                                              | Negative regulation of juvenile hormone biosynthetic process; lipase activity | NA           |
|                     | Aclox57D-d                                                            | Lipid biosynthetic process        | Steroid biosynthesis |
|                     | Gba1a                                                                | Lipid catabolic process           | Alpha-linolenic acid metabolism |
|                     | jhamt                                                                | Hormone metabolic process         | Insect hormone biosynthesis |
|                     | rdgA                                                                 | Phosphatidylinositol biosynthetic process | NA           |
|                     | Pxt, CG8834                                                           | Lipid biosynthetic process        | NA           |
|                     | AstA, AstC                                                            | Regulation of hormone levels; isoprenoid biosynthetic process | NA           |
| Downregulated       | CG1441, CG13091, CG10097, CG17560                                     | Wax biosynthetic process          | Peroxisome   |

NA, no KEGG pathway was enriched

Wolbachia Infection Is Associated with More Active Lipid Metabolism in the Host

Lipid metabolism is likely to be critical to the Wolbachia-host relationship. Both Wolbachia and insects lack cholesterol biosynthesis genes, and the Wolbachia genome also lacks fatty acid synthesis genes, so there is likely to be resource competition between Wolbachia and the host [49]. Our study showed that several genes involved in lipid synthesis were significantly upregulated in DmelW. The process of fatty acid synthesis was significantly upregulated in DmelW, which may supply fatty acids due to a resource shortage caused by competition with Wolbachia. Two studies also found that Wolbachia...
abundance was correlated with increased odd-chain fatty acids and increased mRNA expression of fatty acid synthase [4, 53]. At the same time, we found that several intracellular cholesterol transport genes were significantly upregulated in DmelW, including Npc1b, Npc2f, and Npc2d, which may lead to changes in intracellular cholesterol transport. Cholesterol is vital for membrane stability and cellular signaling in insects. Wolbachia replication is also cholesterol-dependent, as cholesterol-rich host membranes are required to form the vacuole surrounding each bacterium [54], so there is likely to be resource competition between Wolbachia and the host, resulting in accelerated cholesterol transport in the host. Cholesterol also plays an important role in pathogen blockage by Wolbachia. Previous studies have reported that Wolbachia can regulate intracellular cholesterol transport to resist DENV infection [55].

In contrast, some peroxisome-related genes, including CG17560, CG13091, CG14097, and CG1441, were significantly downregulated in DmelW. GO molecular functional analysis showed that these genes have fatty-acyl-CoA reductase (alcohol-forming) activity and are involved in the biosynthesis of insect cutin and wax. This result further indicated that infection with Wolbachia may influence the lipid metabolism of the host in various ways.

**Wolbachia Infection Is Associated with High Expression Levels of Innate Immunity Genes in Native Host**

When Wolbachia is transferred into a novel host, such as a mosquito, it causes a strong immune response in the host [56]. However, in their native hosts, such as *Drosophila*, Wolbachia does not induce an immune response due to the mutually beneficial relationship [57–59]. Wolbachia can also improve the native host’s resistance to pathogens in other ways [55, 60]. However, one study showed that Wolbachia could induce an innate immune response in *D. melanogaster* adult testes [26].

Our results showed that the presence of Wolbachia was related to enhanced immune responses in the testes of its native host, including multiple genes in the Toll and IMD pathways, such as the protein encoded by the DptB gene is an antimicrobial peptide induced by the IMD signaling pathway that promotes resistance to gram-negative bacterial infection [61]. *Drs* and *Drsl3* encode antimicrobial peptides under the control of the Toll signaling pathway induced by fungal infection [62]. Although Wolbachia is surrounded by the host membrane, it has a nearly complete peptidoglycan precursor lipid II synthesis pathway [63]. Wolbachia also encodes an amidase (AmiD) that cleaves its own peptidoglycan to evade the host immune response [64]. We speculate that, due to the higher density of Wolbachia in the testes compared to other tissues, during the rapid proliferation of Wolbachia, it is impossible for it to completely evade the host immune system, resulting in immune recognition. For example, the expression of the peptidoglycan recognition protein gene PGRP-SC2 is significantly higher in Wolbachia-infected testes, and PGRP-SC2 can negatively regulate the IMD signaling pathway by hydrolyzing peptidoglycan, preventing activation of the constitutive IMD pathway, thereby maintaining the balance between immune tolerance and immune response in Wolbachia infection [65].

**Wolbachia Infection Is Not Associated with Oxidative Stress in the Native Host**

In addition to the above results showing that Wolbachia may affect the host’s Toll and IMD pathways, Wolbachia may also affect the level of reactive oxygen species (ROS) in the host [59, 66]. ROS is a natural by-product of oxidative phosphorylation, and it can cause severe cell damage. However, ROS also plays an important role in immune response, not only participating in the transmission of immune signals but also directly killing pathogenic microorganisms [67]. Zug et al. reported that there is a close relationship between Wolbachia and ROS in the host. In a novel host, Wolbachia induces ROS production, resulting in significant upregulation of host antioxidant genes. However, in their native hosts, Wolbachia induces not only ROS production and oxidative stress but also the expression of antioxidant genes (from Wolbachia, the host, or both) to restore oxidative homeostasis [59]. The study by Molloy et al. on ROS in naturally infected versus antibiotic-cured *Aedes albopictus* supports this hypothesis. They found that Wolbachia infection status had no significant effect on ROS and antioxidant enzyme gene expression levels in *A. albopictus*. The author speculated that Wolbachia might not enhance host resistance to pathogens through the ROS-induced immune pathway [68]. We hypothesize that Wolbachia does not induce oxidative stress in their native hosts. To verify this hypothesis, we detected the expression levels of antioxidant genes in Wolbachia-infected and uninfected *D. melanogaster* adult testes. We found that the expression levels of several genes related to ROS degradation were not significantly different between DmelW and DmelT testes, which was consistent with the results of Molloy et al. [68]. Our results indicate that Wolbachia infection does not induce oxidative stress in its native hosts at least in the adult stage.

In summary, our RNA-seq data collected from adult *D. melanogaster* testes showed numerous DEGs between Wolbachia-infected and Wolbachia-free samples, and these genes are mostly involved in carbohydrate metabolism, lysosome, lipid metabolism, immune response, and peroxisome, and no differentially expressed genes putatively associated with spermatogenesis were discovered. These data provide useful molecular information for the study of Wolbachia-host...
intracellular relationships. Subsequent analysis of transcriptome data in Drosophila ovaries would be helpful to further understand the differences in Wolbachia-host molecular interactions between male and female hosts.

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Author Contribution Jinhua Xiao and Dawei Huang conceived the study. Weihao Dou analyzed the data, performed QPCR verification, and wrote the paper. Yunheng Miao provided valuable suggestions on the revision of the paper and the use of some software. All authors have read and approved the manuscript.

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Data Availability The datasets generated and analyzed during the current study are available in the SRA database at NCBI, with the accession number of PRJNA639180.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Written informed consent for publication was obtained from all participants.

Conflict of Interest The authors declare no competing interests.

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