



## Research

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# Single-cell transcriptome sequencing reveals *Wolbachia*-mediated modification in early stages of *Drosophila* spermatogenesis

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*Wolbachia* are the most widely distributed intracellular bacteria, and their most common effect on host phenotype is cytoplasmic incompatibility (CI). A variety of models have been proposed to decipher the molecular mechanism of CI, among which the host modification (HM) model predicts that *Wolbachia* effectors play an important role in sperm modification. However, owing to the complexity of spermatogenesis and testicular cell-type heterogeneity, whether *Wolbachia* have different effects on cells at different stages of spermatogenesis or whether these effects are linked with CI remains unknown. Therefore, we used single-cell RNA sequencing to analyse gene expression profiles in adult male *Drosophila* testes that were infected or uninfected by *Wolbachia*. We found that *Wolbachia* significantly affected the proportion of different types of germ cells and affected multiple metabolic pathways in germ cells. Most importantly, *Wolbachia* had the greatest impact on germline stem cells, resulting in dysregulated expression of genes related to DNA compaction, and *Wolbachia* infection also influenced the histone-to-protamine transition in the late stage of sperm development. These results support the HM model and suggest that future studies on *Wolbachia*-induced CI should focus on cells in the early stages of spermatogenesis.

## 1. Introduction

The intracellular symbiotic bacteria of the genus *Wolbachia* are widely distributed in arthropods and nematodes and are mainly located in the testes and ovaries of arthropod hosts [1,2]. *Wolbachia* can manipulate host reproduction in several ways to enhance their own maternal transmission, and the best-studied host phenotype induced by *Wolbachia* is cytoplasmic incompatibility (CI), in which mating between *Wolbachia*-infected males and uninfected females leads to embryonic lethality [3]. Numerous studies have discovered that CI is related to zygotic interphase defects in de novo nucleosome assembly and replication, leading to delayed activation of the cell cycle kinase Cdk1 and improper chromosome condensation [4–6].

Although the molecular mechanism of CI has not been fully deciphered, a series of models have been proposed. The earliest proposed CI model was the 'mod-resc' (modification/rescue) model, which hypothesizes that CI is caused by the modification of sperm by a factor of *Wolbachia* and that another factor derived from *Wolbachia*-infected eggs can rescue this modification [7]. Over the past few years, *Wolbachia*-encoded CI factors (CifA and CifB) have been identified that appear to be the key to explaining the processes of *Wolbachia*-induced CI and rescue [8,9]. Correspondingly, a variety of improved CI models based on the 'mod-resc' model have been proposed, including the HM (host modification) [5,10] and TA (toxin-antidote) [11] models. The HM model assumes that Cif proteins can modify sperm and that this modification leads to the segregation failure of paternally derived chromosomes and the phenotype of CI in fertilized eggs, unless this modification is reversed or removed

by CifA derived from *Wolbachia*-infected females [10]. The TA model assumes that in embryos, CifB from the male gamete plays the role of toxin, which can lead to the CI phenotype, while these defects can be rescued by the CifA antidote binding CifB in the embryo [11].

Some insightful results about CI have emerged recently, providing evidence supporting one or more predictions of these models. Some evidence seems to support the theory of toxin–antidote binding in the TA model, such as *in vitro* pull-down experiments showing that CifA can bind to CifB [8], and high-resolution structures of CifA and CifB also support the binding of the two proteins [12]. Studies have shown that CifB is sufficient to induce CI in males, and this CifB-induced sterility is rescued by CifA expression in females [13,14]. However, whether this rescue is due to the binding of CifA and CifB in the embryo under the assumption of the TA model is still unclear as no *in vivo* data in the host embryos has yet been obtained. Alternatively, the co-expression of CifA and CifB may be the key to inducing CI, according to the two-by-one or HM model [10,15]. For example, in some cases, co-expression of CifA and CifB is necessary for the induction of CI in transgenic male flies [8,9,16], and transmission of the same *Wolbachia* strain into different hosts may induce CI phenotypes to different degrees [17,18], which suggests that host factors play an important role in *Wolbachia*-induced CI. Moreover, many studies have indicated the importance of the establishment of *Wolbachia* modification in the testes during the induction of CI, consistent with the HM model. For example, for some CI-inducing *Wolbachia* strains, the host sperm cells in the pre-fertilization stages show abnormal morphology or lower competitiveness, suggesting that the sperm cells have been modified [19–21]. Importantly, a paper recently published by Kaur *et al.* [22] strongly supported the HM model by providing evidence that Cif proteins localize to the nuclear DNA of the host in the process of spermatogenesis and cause abnormal histone retention in elongating spermatids and protamine deficiency in mature sperm.

Based on the HM model, many researchers have carried out various omics studies on the putative modification of testes by *Wolbachia* and identified multiple host genes and metabolic pathways that may be closely related to CI [23–30]. However, owing to the complexity of spermatogenesis and the heterogeneity of cell types in the testes, we still do not know whether *Wolbachia* have different effects on cells at different stages of spermatogenesis or whether these effects may be associated with CI. For example, studies have shown that the distribution pattern of *Wolbachia* in *Drosophila* testicular cells is uneven, *Wolbachia* are present in developing spermatocytes and spermatogonia but absent in mature sperm, so the CI-related effects of *Wolbachia* may start occurring early in sperm development [31–34]. Furthermore, in the parasitic wasp *Nasonia vitripennis*, *Wolbachia*-induced CI is close to 100%, but *Wolbachia* are found in only 28% of developing sperm, suggesting that *Wolbachia* may also modify uninfected germ cells by secreting effectors [34]. Based on the above evidence, we speculate that the effects of *Wolbachia* on testicular cells at different stages of spermatogenesis are complex, and the bacteria may have different modification effects on different cell types.

While previous studies have shown the complex impacts of *Wolbachia* on spermatogenesis that can result in

downstream sperm defects [17], a deeper investigation of these defects is needed to confirm their connection to CI. In this study, we employed single-cell RNA sequencing (scRNA-seq) of 1-day-old adult male *Drosophila melanogaster* testes infected or uninfected with *Wolbachia*. We attempted to compare the effects of *Wolbachia* on gene expression profiles in different cell types of *Drosophila* testes in order to identify the cell type that *Wolbachia* mainly affects and to explore the host metabolic pathway and gene alterations. Our results showed that *Wolbachia* have the strongest effect on cells in the early stage of spermatogenesis, and the alteration in the process of DNA compaction in the early stage of sperm development may be related to the establishment of HM in the mechanism of CI.

## 2. Methods

### (a) Fruit fly rearing

*Drosophila melanogaster* naturally infected *Wolbachia* strain *wMel* was available in our laboratory; *Wolbachia*-uninfected *D. melanogaster* strains were established following the previously reported method [28]. All flies were reared on standard *Drosophila* cornflour medium at 25°C with a photoperiod of 14 h L : 12 h D (light : dark) and 40% relative humidity [35]. Each strain had been cultivated for more than 40 generations before this experiment.

### (b) Embryo hatch rate experiment

We performed the CI test as previously described by Yamada *et al.* [36]. We crossed 3-day-old virgin female flies (*Wolbachia*-infected or uninfected) with 1-, 3- and 5-day-old male flies (*Wolbachia*-infected or uninfected) to examine the effect of male age on the strength of CI. We used agarose/apple juice medium and smeared fresh yeast on the surface for flies to lay eggs. First, a single 3-day-old virgin female was put into each plastic vial containing medium. After 4 h of acclimation, a single virgin male was added to each vial. After mating for approximately 6 h, the females were transferred to a new vial containing fresh medium and incubated at 25°C for 24 h for egg laying. Then, the females were removed, and the eggs were counted immediately; females that laid fewer than five eggs were discarded from the experiment. All vials were incubated at 25°C for another 48 h, and the number of remaining unhatched eggs was counted. Embryo hatch rates were calculated by determining the ratio of the number of hatched eggs to the total number of eggs.

### (c) Microbiome analysis

One-day-old *Wolbachia*-infected and uninfected male flies were used for intestinal genome extraction and 16S rRNA gene amplicon sequencing (see electronic supplementary material, text for details). FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used for raw read quality control, and fastp was used to quality filter the raw data with the following parameters: fastp -q 20 -u 20 -n 5 -c. We used DADA2 (v.1.24.0) to produce amplicon sequence variants (ASVs) based on denoising algorithms [37], used the silver138 database to annotate ASV sequences, used the R package ‘phyloseq’ to normalize the ASV table to eliminate any bias due to differences in the sampling sequencing depth, and used the `syntax_summary` function from ‘usearch’ to determine the frequencies of each named taxon at different ranks. We performed principal component analysis (PCA) using the ‘prcomp’ R package. We used ‘vegdist’ to calculate Bray–Curtis dissimilarities and performed principal coordinates analyses (PCoA) by using the ‘ape’ R package.

Permutational multivariate analysis of variance (PERMANOVA) was conducted using the `adonis2` function in the 'vegan' R package to analyse the significance of the community differences. Data link: <https://doi.org/10.5061/dryad.stjq2c5w>.

#### (d) Preparation of single-cell suspensions

One-day-old *Wolbachia*-infected and uninfected males were used. For each group, 20–30 pairs of adult male testes were dissected one by one in cold EBSS (NaCl 6.8 g l<sup>-1</sup>, NaH<sub>2</sub>PO<sub>4</sub> 0.112 g l<sup>-1</sup>, KCl 0.4 g l<sup>-1</sup>, D-glucose 1 g l<sup>-1</sup>, NaHCO<sub>3</sub> 2.2 g l<sup>-1</sup>, without calcium, magnesium and phenol red). Cells were dissociated by lysis solution (EBSS containing 4 mg ml<sup>-1</sup> elastase and 2.5 mg ml<sup>-1</sup> type IV collagenase) and filtered for purification. Libraries were prepared with the 10X Genomics chromium 3' kit, and sequenced on a MGISEQ2000 instrument (BGI, Shenzhen, China) using 100 nucleotide (nt) paired-end sequencing, with at least 7 million reads obtained per library. A detailed description of the methods can be found in electronic supplementary material, methods.

#### (e) Downstream single-cell RNA sequencing analysis

The FASTQ file of each sample (WinfM and WuninfM) was used for Cell Ranger (v.6.1; <https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest>) analysis. This output was then imported into the Seurat (v.4.0.1) R package [38,39] for quality control and downstream analysis of our scRNA-seq data. Data link: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA788731?reviewer=n7svq5k71ohhepvg14k41k4hhs>.

#### (f) Identification of cell types in single-cell RNA sequencing data

Specific markers in each cluster were identified by the 'FindAllMarkers' function in the 'Seurat' package' (options: `only.pos = TRUE`, `min.pct = 0.25`, `logfc.threshold = 0.25`). All predicted marker genes for each cell type are shown in electronic supplementary material, table S1. We used well-known reported marker genes for cell type identification [40,41].

#### (g) Functional enrichment analysis of differentially expressed genes

We identified differentially expressed genes (DEGs) for each cell type between *Wolbachia*-infected and uninfected samples through the 'FindMarkers' function in 'Seurat' (`logfc.threshold = 0.25`, `min.pct = 0.25` and `test.use = wilcox`), and  $|\log_2FC| > 0.25$  and  $p\text{-value} < 0.05$  were considered statistically significant. For the DEGs of each cell type, the 'clusterProfiler' (v.3.18.1, [42]) was used with `enrichGO` for functional analysis, with Fisher's exact tests (two-sided) performed, and  $p$ -value adjusted by the Benjamini–Hochberg (BH) procedure, with adjusted  $p$ -value ( $p\text{-adjust}$ )  $< 0.05$  defined as statistically significant.

#### (h) Pseudotime inference analysis and identification of differential gene expression patterns

The Monocle3 package (v.1.0.0) [43] was used to analyse single-cell trajectories to reveal differential gene expression patterns associated with cell-state transitions. We reconstructed the expression pattern of some selected genes of interest involved in spermatogenesis or CI in germ cells of WuninfM and WinfM samples along pseudotime, and genes with significant divergence in expression dynamics are displayed. Details are provided in electronic supplementary material, text.

#### (i) Distribution of *Wolbachia* reads in single-cell RNA sequencing data and annotation of *Wolbachia* genes

To detect the presence of *Wolbachia* genes in scRNA-seq data, we first selected *Wolbachia* reads from scRNA-seq data of the WinfM sample and calculated the density of *Wolbachia* reads by matching each read with a 10× barcode to each cell type. Then, we used Trinity (v.2.8.5; <https://github.com/trinityrnaseq/trinityrnaseq/releases>) to assemble and eggNOG-mapper (<http://eggnog-mapper.embl.de/>) to annotate *Wolbachia* reads in each cell type. As a control, we also mapped scRNA-seq reads of the WuninfM sample to the *Wolbachia* wMel reference genome, and no *Wolbachia* reads were detected.

#### (j) *cif* gene expression assays

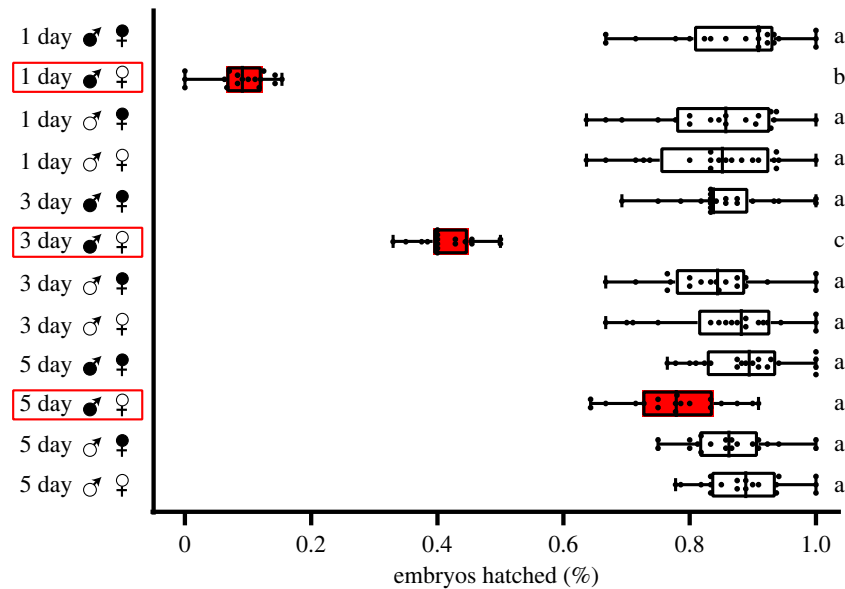
To identify *cif* genes in the *Wolbachia* genome, DNA was extracted from *Wolbachia*-infected male flies using a QIAGEN DNeasy Blood & Tissue DNA kit (Qiagen, Germany), with uninfected males as the negative control. *cif*-specific primers were used to amplify *cif* gene fragments (WD0631-F: ATAAAGGCGTTTCAG-CAGGA, WD0632-R: TTGCCAGCCATCATTCTATAA) [44]. The PCR mixture contained forward primer (1.25 µl), reverse primer (1.25 µl), Q5 High-Fidelity 2X Master Mix (12.5 µl; New England Biolabs, Ipswich, MA, USA), DNA template (2 µl) and nuclease-free water (8 µl). The PCR programme used was as follows: 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 54°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were detected with 1% agarose gel electrophoresis and Sanger sequencing.

Quantitative reverse transcription PCR (qRT-PCR) was performed to determine the relative expression dynamics of *cif* genes with increasing *Drosophila* male age. One-, 3- and 5-day-old virgin male flies were collected for RNA extraction (siblings from hatch rate assays). The male testis tissues were dissected in cold phosphate-buffered saline (PBS), and 10 pairs of testes were placed into a single 1.5 ml tube with 500 µl of TRIzol. We used the TransZol Up Plus RNA Kit (TransGen, Beijing, China) to extract and purify RNA. First-strand synthesis of cDNA was completed by using TransScript First-Strand cDNA Synthesis SuperMix with random primers (TransGen, Beijing, China). The specific primers used for the *Wolbachia* *cif* genes for qPCR were as described by Shropshire *et al.* [33], and all the primers are listed in electronic supplementary material, table S2. qPCR was performed using a CFX96 system (Bio-Rad) with PerfectStart Green qPCR SuperMix (TransGen, Beijing, China). The qPCR programme was as follows: 50°C for 2 min, 95°C for 2 min, followed by 39 cycles of 95°C for 15 s, 54°C for 15 s and 72°C for 20 s, and a final dissolution step. All samples were tested in triplicate. The *Wolbachia* *ftsZ* and *D. melanogaster*  $\beta\text{-spec}$  genes were used as reference genes for normalization. The fold change was calculated as  $2^{-\Delta\Delta Cq}$ .

### 3. Results

#### (a) *Wolbachia* induced the strongest cytoplasmic incompatibility in 1-day-old males

To test the CI strength of the *Wolbachia* strain wMel, we performed a hatch rate experiment on male flies of different ages. The results showed that the strength of CI induced by *Wolbachia* was closely related to male age, since *Wolbachia* induced almost 100% CI in 1-day-old males, while 5-day-old males expressed no CI (figure 1). We thus focused on 1-day-old males for subsequent studies.  $p$ -values are reported in electronic supplementary material, table S3.



**Figure 1.** Embryo hatch rate analyses showing the effect of *Wolbachia*-induced cytoplasmic incompatibility (CI), which decreased with male age. The horizontal axis shows the hatch rate, and the vertical axis shows different cross groups (days represent male age, black solid circles represent *Wolbachia*-infected samples, and open circles represent uninfected samples). CI crosses are coloured red, boxplots represent median and interquartile ranges. Letters to the right represent statistically significant differences based on  $\alpha=0.05$  calculated by Kruskal–Wallis test followed by Dunn’s test with corrections for multiple comparisons between all groups; crosses with different letters are significantly different. These data demonstrate that 1-day-old males show the strongest CI.

### (b) The gut bacterial community structures of male flies were highly correlated with *Wolbachia* infection

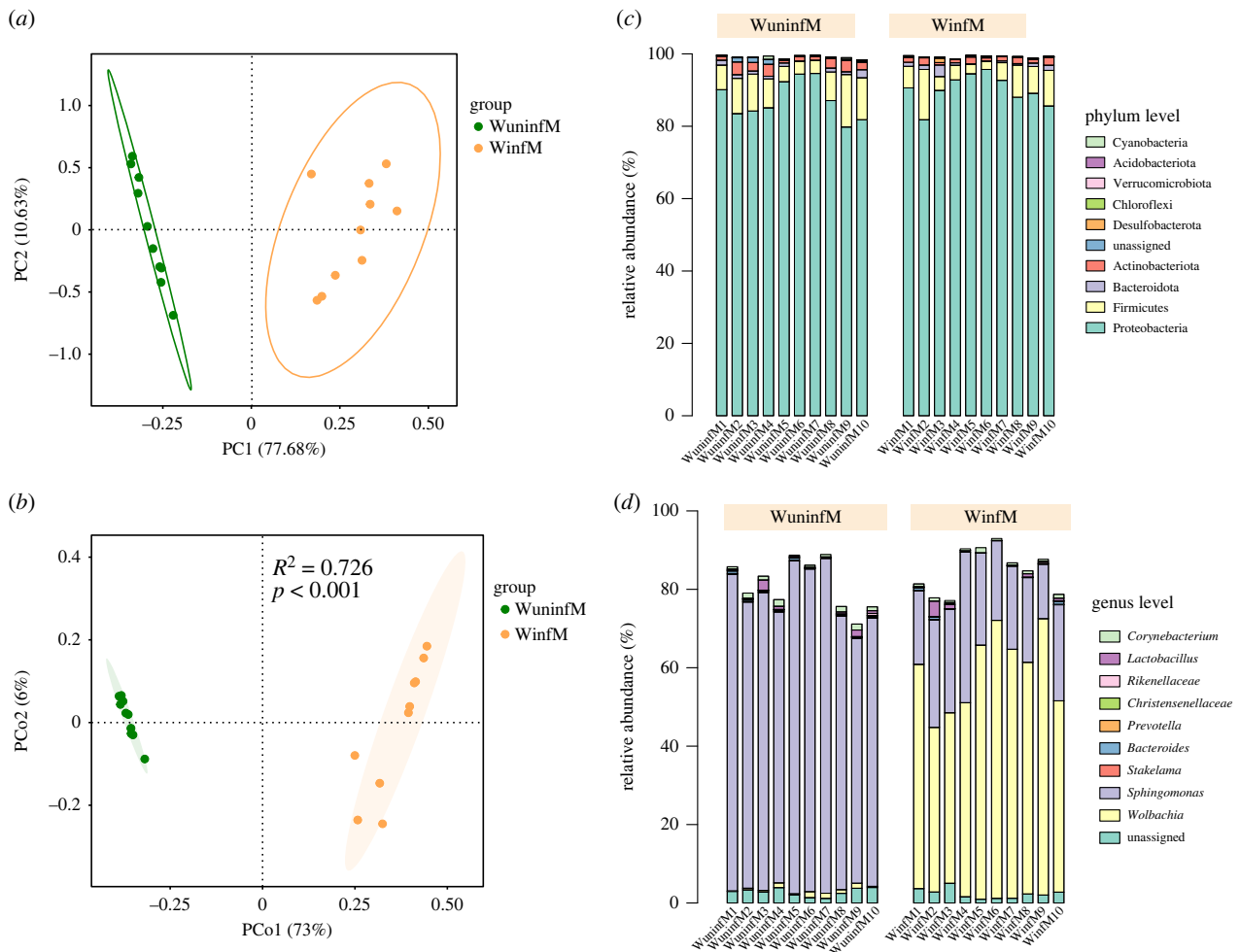
Uninfected *Drosophila* strains are mainly obtained through antibiotic treatment. However, many studies have shown that antibiotic treatment can significantly change the host’s microbiome [45–48], which may lead to changes in host gene expression. To detect changes in the microbiome of the uninfected *Drosophila* strain after recovery from antibiotic treatment for more than 40 generations, we performed microbiome analyses. PCA showed that *Wolbachia*-infected and uninfected samples were well divided into two clusters. Consistent with this, the two *Drosophila* strains were also significantly separated in the PCoA (PERMANOVA:  $R^2=0.728$  and  $p<0.001$ ) (figure 2a,b). To further determine which bacterial group in *Drosophila* was changed owing to the long-term differences in *Wolbachia* infection and non-infection, we compared the differences in the bacterial community based on the top 10 bacterial groups at the phylum and genus levels. There was no significant difference in abundance between the groups at the phylum level (figure 2c). However, at the genus level, *Wolbachia* was the dominant genus in the *Wolbachia*-infected samples, and *Wolbachia* was also the genus with the largest difference between the two groups (figure 2d). Therefore, we speculate that the change in host gene expression is mainly related to *Wolbachia* infection.

### (c) Single-cell expression atlas and cell typing in adult *Drosophila* testes infected and uninfected with *Wolbachia*

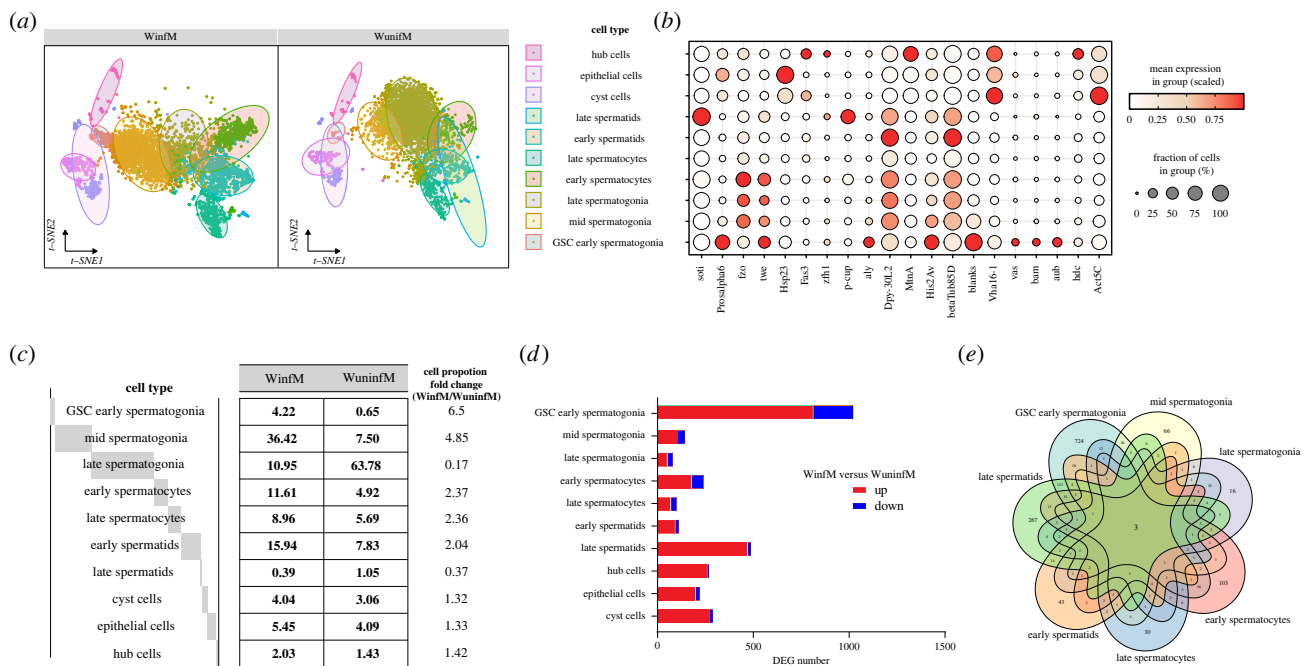
To understand the modification effects of *Wolbachia* on different cell types in testes, we performed scRNA-seq on *Wolbachia*-infected (WinfM) and uninfected (WuninfM) one-day-old male *Drosophila* testes. After the initial quality control

step (see Methods), we acquired a total of 8372 high-quality cells, with 4385 cells from WinfM and 3987 from WuninfM samples. To explore the cellular compositions, we applied PCA to DEGs across all cells for dimensionality reduction. We further used unsupervised graph clustering to partition the cells into 10 clusters and visualized them *via* *t*-distributed stochastic neighbour embedding (*t*-SNE) (figure 3a). We generated cluster-specific marker genes via differential gene expression analysis and used previously reported marker genes to identify the cell types. These well-known cell type markers, such as *soti* for late spermatids, *prosalpha*, *aly* and *bam* for germline stem cells (GSCs), *hdc* for hub cells, and *Act5C* for somatic cells [36,37], were used to determine the cellular identity of the clusters (figure 3b). We finally identified a total of 10 cell types, with seven germ cell types, including GSC early spermatogonia, mid spermatogonia, late spermatogonia, early spermatocytes, late spermatocytes, early spermatids and late spermatids, and three somatic cell types, hub, cyst and epithelial cells. The relative expression of markers in all cell types is shown in electronic supplementary material, table S1.

Following initial cell-type characterization, we calculated the percentage of each cell type in both samples. In the WuninfM sample, the vast majority of cells (63.78%) were in the late spermatogonia stage, and the cell type with the lowest proportion (0.65%) was GSC early spermatogonia, while in the WinfM sample, the cell type with the highest proportion (36.42%) was mid spermatogonia and the lowest proportion was observed in late spermatids (0.39%) (figure 3c). We further compared the differences in the proportion of each cell type between the two samples and found that the proportions of most cell types varied greatly upon *Wolbachia* infection. For germ cells, the proportions of GSC early spermatogonia, late spermatogonia, mid spermatogonia, early spermatocytes and late spermatids differed greatly between the WinfM and WuninfM samples, among which the proportions of GSC early spermatogonia, early



**Figure 2.** Gut bacterial diversity analyses of *Wolbachia*-infected (WinfM) and uninfected (WuninfM) *Drosophila* males. (a) Principal component analysis (PCA) of 16S rRNA gene-sequencing data of gut microbes obtained from *Wolbachia*-infected and uninfected male flies. (b) Principal coordinate analysis (PCoA) based on Bray-Curtis distances between both groups, with  $R^2$  and  $p$ -values derived from the adjusted PERMANOVA models. (c,d) Top 10 relative abundances of bacteria, showing bacterial community structure at the phylum and genus levels.



**Figure 3.** Diverse cell types identified in *Drosophila* testes from 1-day-old males by single-cell RNA sequencing (scRNA-seq) analysis. (a) A  $t$ -distributed stochastic neighbour embedding ( $t$ -SNE) visualization and clustering of WuninfM and WinfM samples. (b) Dot plot of scaled expression of marker genes in each cell type. For each dot, the size refers to the proportion of cells expressing a gene, and the colour represents the calculated scaled expression value. (c) Proportions of each cell type in the WinfM and WuninfM samples and cell proportion fold changes between the two samples. (d) Numbers of differentially expressed genes (DEGs) between WinfM and WuninfM samples in each cell type ( $|\log_2$ -fold change  $\geq 0.25$  and  $p$ -value  $< 0.05$ ); up: upregulated in WinfM, down: downregulated in WinfM. (e) Venn diagram showing only three core-regulated DEGs among all germ cell types.

spermatocytes and mid spermatogonia were higher in the WinfM sample, and the proportions of late spermatogonia and late spermatids were lower in the WinfM sample, than in the WuninfM sample (figure 3c). For the three somatic cell types, the proportions in the WinfM sample were slightly higher than those in the WuninfM sample, and the difference was not as significant as those of germ cells (figure 3c). All these findings indicate that several cell types are affected by *Wolbachia* infection, suggesting that the cell-type-specific effects of *Wolbachia* deserve further exploration.

#### (d) Cell-type-specific aberrant gene expression in *Wolbachia*-infected samples

Next, we compared transcriptome profiles between WinfM and WuninfM samples for each cell type (electronic supplementary material, table S4). We found that the number of upregulated genes was higher than the number of downregulated genes in all cell types in the WinfM sample compared with the WuninfM sample; moreover, the number of DEGs varied greatly among different types of germ cells. GSC early spermatogonia contained the largest number of DEGs among all germ cells, followed by late spermatids and early spermatocytes, suggesting that *Wolbachia* might have a greater effect on these cell types (figure 3d). In addition, a total of 1653 DEGs were found in all germ cells; however, only three of them (*CG43800*, *hydra* and *Scfp*) displayed differential expression in all germ cells (figure 3e), implying that the majority of the DEGs were cell-type-specific. These results indicate the cell-type-specific impact of *Wolbachia* on gene expression in *Drosophila* testes.

#### (e) Functional enrichment analyses of cell-type-specific differentially expressed genes revealed differential effects of *Wolbachia* on different cell types

To further clarify the function of DEGs between WinfM and WuninfM samples, we performed functional enrichment analysis on DEGs of each cell type (electronic supplementary material, table S5). Among germ cells, GSC early spermatogonia, late spermatids and early spermatocytes showed larger changes in cell proportion and more DEGs than other cell types, which led us to focus on these three germ cell types in subsequent analyses. For the three germ cell types, we found that the upregulated DEGs in the infected samples were mostly enriched in microtubule-based, ubiquitin-dependent protein catabolic, proteolysis involved in protein catabolic, reproduction-related and ATP metabolic processes. The downregulated genes in GSC early spermatogonia were significantly enriched in translation, ribonucleoprotein complex biogenesis, regulation of cellular response to stress, nucleus organization and male gamete generation processes (figure 4a).

To further target the key genes in the above biological processes, we selected genes with three criteria (fold change, *p*-value, previously reported related to CI) and determined their differential expression patterns in the three types of cells; these genes were mainly differentially expressed in GSC early spermatogonia (figure 4b). Then, we performed protein–protein interaction network analysis on these genes and found that the interaction network of these genes is mainly divided into two clusters: protein catabolism-related

genes (such as multiple testis-specific proteolysis genes) and reproduction-related genes (such as *His3.3A*, *His3.3B*, *ProtA*, *ProtB*, *Nap1* and *ATPsynbetaL*). Therefore, the DEGs involved in these two biological processes in germ cells, especially in GSCs, may be the key host factors for *Wolbachia*-mediated modification of sperm (figure 4c).

Interestingly, the functional enrichment results of DEGs in the three somatic cell types were quite different from those of germ cells. Somatic DEGs were mainly involved in transcription, cellular secretion, cell redox homeostasis, NADH metabolism, cellular response to toxic substances and other processes (figure 4d). These results indicate that *Wolbachia* have distinct interactions with somatic and germ cells.

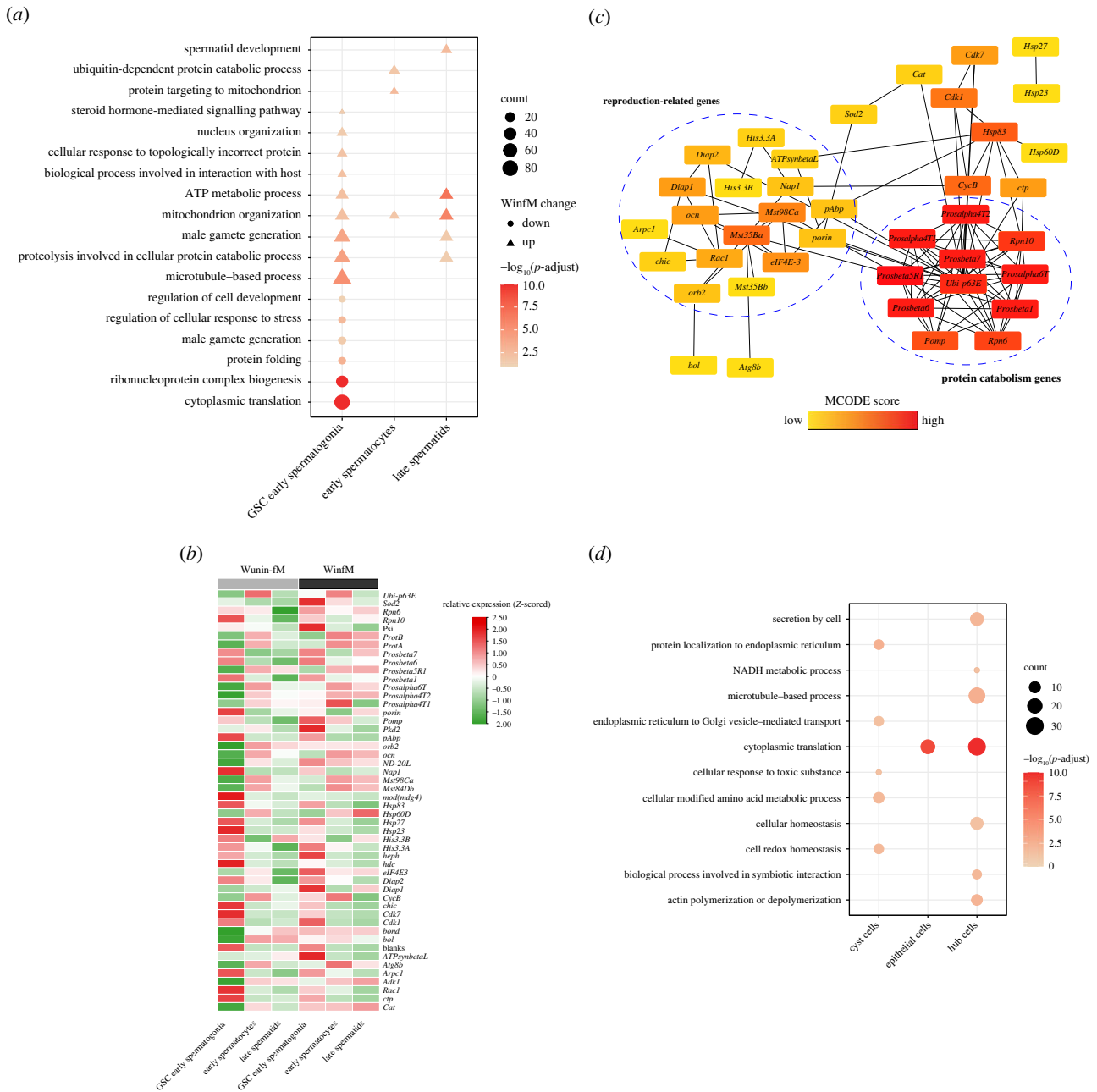
#### (f) *Wolbachia* infection caused various differentially expressed genes to exhibit different dynamic expression patterns during spermatogenesis

Different genes tend to have different expression patterns at different stages of sperm development. To decipher the transcriptomic dynamics during spermatogenesis, we performed pseudotime analysis on the germ cells of the WinfM and WuninfM samples using Monocle3 and found that both samples had similar cell development trajectories (electronic supplementary material, figure S1).

We reconstructed the dynamic expression patterns for the selected DEGs as shown in figure 4b. We found that multiple genes, such as *Nap1*, *ProtA*, *ProtB*, *CycB*, *bol*, *Diap1*, *Pomp*, *bond*, *sod2*, *orb2*, *pAbp*, *ATPsynbetaL*, *Pkd2*, *Prosalpha4T2*, *Mst84Db* and *eIF4E3*, exhibited significant dynamic differences in expression between the two samples (figure 5a and electronic supplementary material, figure S2). Among them, six candidate genes, *Nap1*, *ProtA*, *ProtB*, *orb2*, *Diap1* and *ATPsynbetaL*, were selected according to two independent criteria—the differences in expression patterns along the trajectories and the fold changes in expression levels between the two samples. These genes were related to male gamete generation; they showed significant dynamic expression differences between WinfM and WuninfM samples in early spermatogenesis; however, in late spermatogenesis, the expression patterns converged (figure 5b–g). These patterns suggest that *Wolbachia* have a greater effect on the early stage of spermatogenesis. In addition, we found that the expression levels of *ProtA* and *ProtB* were significantly increased at the later stage of sperm development (figure 5c,d).

#### (g) The presence of active *Wolbachia* genes in different cells suggested differential interaction of *Wolbachia* with different cell types

Based on the HM model that *Wolbachia*-induced CI depends on the modification of sperm by *Wolbachia* effectors, such as CifA and CifB, we attempted to determine the expression pattern of *Wolbachia* genes from the scRNA-seq data. We assumed that the number of detected *Wolbachia* sequences was positively correlated with the number of active *Wolbachia* cells. Based on this assumption, we discovered that the distribution of *Wolbachia* among different cells was uneven. The highest *Wolbachia* density was in somatic cells, mostly in hub cells, while among germ cells, the highest density was in GSC early spermatogonia, and there was a decreasing trend in *Wolbachia* density during the process of

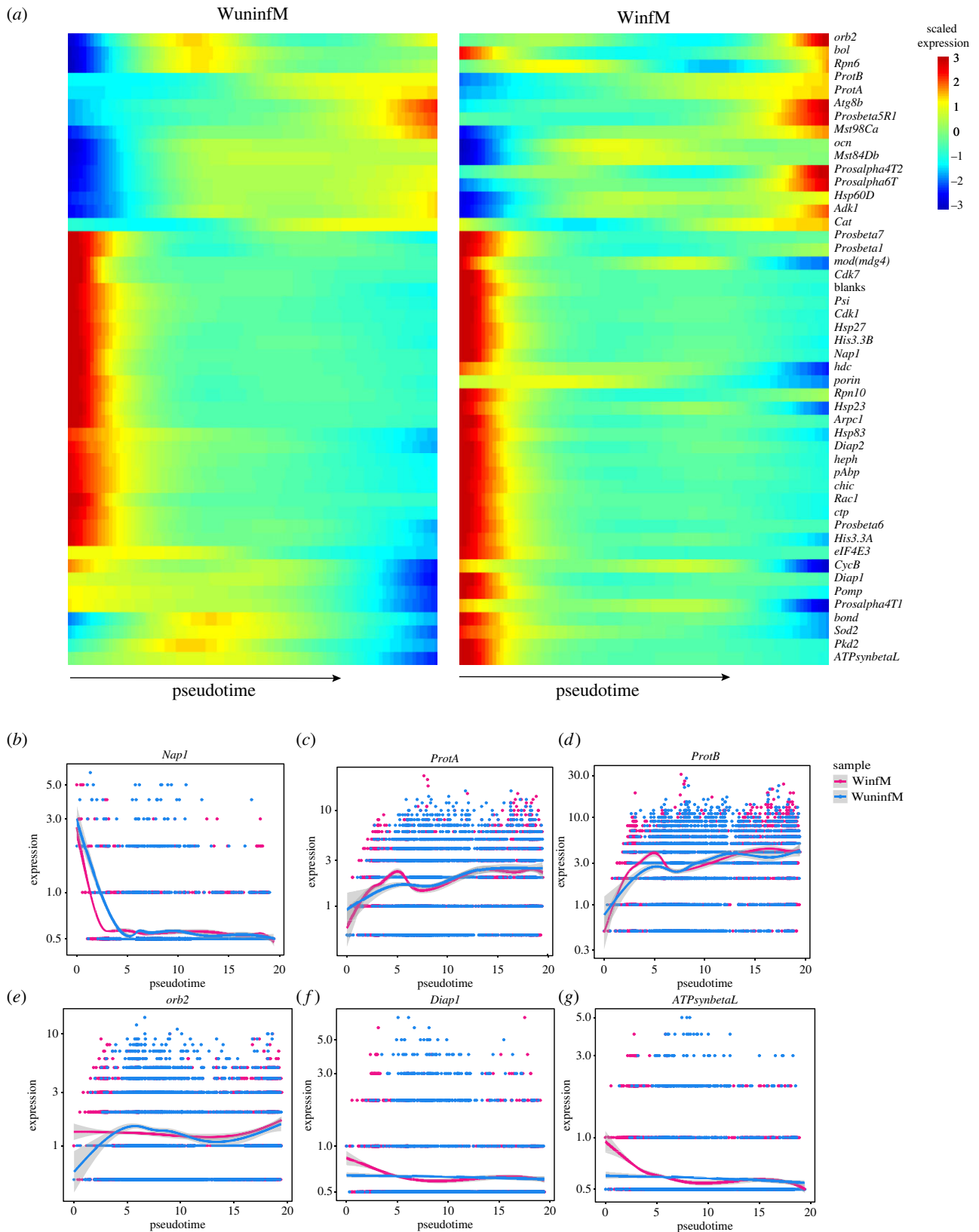


**Figure 4.** Functional analysis of the cell-type-specific differentially expressed genes (DEGs) between WinfM and WuninfM samples. (a) Enriched biological processes of DEGs in the three germ cell types. Triangles represent the enriched GO terms of upregulated genes in WinfM, while dots represent the enriched GO terms of downregulated genes in WinfM; Fisher's exact test (two-sided) performed. The  $p$ -values were adjusted by the Benjamini–Hochberg (BH) method, with adjusted  $p$ -value ( $p\text{-adjust}$ ) < 0.05 defined as statistically significant. (b) Relative expression of selected genes in the three germ cell types. (c) Protein–protein interaction network analysis of selected DEGs, showing only connected nodes in the network. The nodes represent the genes, and edges indicate interaction associations between nodes. The MCODE (Molecular Complex Detection) plugin from Cytoscape was utilized to identify highly interconnected clusters from the network. Colours of the dots represent the connectivity index derived from MCODE scores. (d) Enriched biological processes of DEGs in the three somatic cell types; Fisher's exact test (two-side) performed. The  $p$ -values were adjusted by the BH method, with adjusted  $p$ -value ( $p\text{-adjust}$ ) < 0.05 defined as statistically significant.

spermatogenesis (electronic supplementary material, figure S3a). Then, we performed assembly and functional annotation of the *Wolbachia* sequences in each cell type, with a total of 38 *Wolbachia* genes annotated (*cifA* and *cifB* were not detected). We found that the number of *Wolbachia* genes annotated in different cells varied, with the most genes annotated in the hub cells (electronic supplementary material, figure S3b,c). In hub cells, the annotated *Wolbachia* genes were involved in translation, amino acid transport, vesicular transport, cell membrane/cell wall synthesis and other processes (electronic supplementary material, figure S3d), implying that *Wolbachia* proliferation occurs primarily in hub

cells. Interestingly, in several cell types, the *iscU* gene was annotated; this gene encodes a component of the iron–sulfur (Fe–S) cluster scaffold, and Fe–S clusters are important cofactors in the functioning of diverse enzymes involved in iron homeostasis and oxidative stress response. *WD\_1250* expressed in hub cells is also associated with redox homeostasis. Thus, the expression of *Wolbachia* genes with different functions detected in different cell types suggests that *Wolbachia* genes interact differently with different cell types in *Drosophila* testes.

Since we did not detect any expression of *cif* genes from the scRNA-seq data, to further confirm whether there are *cif* genes



**Figure 5.** Cell trajectory analysis of spermatogenesis-related genes. (a) Heatmap showing the expression pattern of selected differentially expressed genes (DEGs) across single cells along pseudotime in WuninfM and WinfM samples. (b–g) Smoothed expression curves of several candidate genes along the trajectories of spermatogenesis in both samples. The blue and pink lines represent the WuninfM and WinfM samples, respectively, and the shaded area around the line represents the 95% confidence interval.

in the *Wolbachia* genome and to measure the expression of *cif* genes, we performed amplification, sequencing and qPCR expression analyses on *cifs*. The *cif* sequences of *Wolbachia* obtained by amplification and sequencing were completely consistent with the *cif* gene reference sequences of *Wolbachia*

*wMel* (electronic supplementary material, figure S4a,b). The expression of *cifA* and *cifB* had no significant correlation with male age, but the expression ratio of *cifA* and *cifB* decreased with male age (not statistically significant) (electronic supplementary material, figure S4c). Therefore, the



expression of *cif* genes does not sufficiently explain the variation in CI strength, which has been confirmed in previous studies [33].

## 4. Discussion

The *Wolbachia*-induced reproductive regulation phenotype of CI has been extensively studied in a variety of insects; however, the molecular mechanism of CI remains controversial. In the present study, through microbiome analysis, we first determined that changes in host gene expression may be mainly associated with *Wolbachia* infection. Next, through single-cell RNA sequencing, our results revealed that *Wolbachia* have the greatest effect on cells in the early stages of sperm development from three perspectives. First, the proportion of early spermatogenesis cells, such as GSC early spermatogonia, in the WinfM sample was significantly higher than that in the WuninfM sample, suggesting that in the *Drosophila* testes, *Wolbachia* infection causes more cells to be retained in the early stages of sperm development. Second, the number of DEGs in GSC early spermatogonia was the largest among all cell types, suggesting that *Wolbachia* may cause more modifications in early spermatogenesis, such as in GSCs. Finally, in the analysis of gene expression dynamics, multiple DEGs related to spermatogenesis or male gamete generation, such as *Nap1*, *Diap1*, *ProtB*, *ProtA*, *orb2* and *ATPsynbetaL*, also showed differential expression patterns along the developmental trajectories between samples of WinfM and WuninfM, since these genes showed a pattern of significant differential expression in the early stage of spermatogenesis and were more similar in the late stages, suggesting that *Wolbachia* mainly affect the early stages of spermatogenesis.

*Wolbachia* not only affect processes related to reproduction in germ cells but also significantly affect the processes of mitochondrial energy metabolism and proteolysis. On the one hand, the upregulated DEGs in the three germ cells were significantly enriched in biological processes related to ATP metabolic processes, and studies have shown that *Wolbachia* infection can significantly enhance host energy consumption, resulting in excessive oxidative stress and intracellular DNA damage, which in turn affects sperm development [49–51]. On the other hand, the ubiquitin proteolysis pathway plays a key role in chromosomal remodelling and is involved in the conversion of histones to protamine during the later stages of spermatogenesis [52], and mutations in some testis-specific proteolysis genes may even lead to defects in sperm nuclear morphology, with histones being not effectively removed [53]. In our results, multiple testis-specific proteolysis genes, including *Prosalpha4T1*, *Prosbeta5R*, *Prosalpha3*, *Prosbeta6*, *Prosbeta7*, *Rpn12* and *Rpn6*, were upregulated in germ cells, especially in GSC early spermatogonia and late spermatids. We speculate that *Wolbachia* can modify the protein catabolic process and mitochondrial energy metabolic process, leading to abnormal sperm cell production.

In addition to the effect on germ cells, the influence of *Wolbachia* on somatic cells cannot be ignored, since hub cells and cyst cells also play a crucial role in the development of germ cells by secreting various signalling molecules [54,55]. Here, we found that *Wolbachia* induced more differential gene expression between WinfM and WuninfM samples

in hub cells and cyst cells than in most germ cells, and in these somatic cells, the DEGs were significantly enriched in processes of translation, cellular response to toxic substances, cellular homeostasis, cell redox homeostasis, NADH metabolic process and secretion by cells, significantly different from the results for germ cells. In addition, we found that the density of *Wolbachia* sequences was the highest in hub cells, indicating that *Wolbachia* metabolic activity may be more active in hub cells, therefore resulting in alterations in many pathways involved in metabolism and cellular homeostasis in these cells.

According to the HM model, *Wolbachia* factors can modify host factors [10], indicating that host factors play an important role in CI. A recent study has shown that *Wolbachia* modifies developing sperm at the canoe stage [22]. Here, we found that the cells of the sperm developmental stage most affected by *Wolbachia* were GSC early spermatogonia and that *Wolbachia*-induced downregulated genes in GSC early spermatogonia were significantly enriched in male gamete generation. Importantly, three genes involved in chromosome condensation, including *Nap1*, *His3.3B* and *mod(mdg4)*, were only downregulated in GSC early spermatogonia, among which *Nap1* encodes a host protein closely related to CI [56,57]. Therefore, we speculate that the DNA compaction process in early spermatogonia is affected by *Wolbachia*. These results are also highly consistent with the newly discovered results showing that the CifA and CifB proteins are localized to nuclear DNA in the early stage of spermatogenesis, indicating that *Wolbachia*-mediated modification of sperm may be established in the early stage of sperm development [22]. Our results also indicate the influence of *Wolbachia* in the later stage of spermatogenesis. During sperm development, histones bind to the nucleosomes in the early stage, being replaced by protamine at later stages for the tight packaging of DNA in the nucleus within the sperm head [58]. We found that the expression levels of the Protamine A (*ProtA*) and Protamine B (*ProtB*) genes were significantly higher in late spermatids than in early germ cells and were significantly upregulated in *Wolbachia*-infected samples. ProtA and ProtB play important roles in chromosome remodelling during the transition from histones to protamine in the late stage of sperm development, where they are integrated into the sperm nucleus, contributing to the formation of condensed sperm chromatin, and mutations in *ProtA* and *ProtB* genes can cause improper nuclear morphology [58–60]. The new results of Kaur *et al.* [22] show that Cifs can cause abnormal histone retention in elongating spermatids and protamine deficiency in mature sperm. Our results showing higher expression of the protamine genes *ProtA* and *ProtB* in late spermatids in *Wolbachia*-infected testes also indicate that the modification of sperm by *Wolbachia* is also closely related to this chromosome remodelling stage.

## 5. Conclusion

Overall, we thoroughly analysed the effects of *Wolbachia* on gene expression in *Drosophila* testes at the single-cell transcriptome level. We identified the metabolic pathways most affected by *Wolbachia* in germ cells, such as reproduction and protein degradation. We also revealed that early spermatogenesis cells are the cell type strongly impacted by *Wolbachia* and recapitulate previous finding that the sperm

DNA compaction is the key target for CI modification. Thus, our data support the hypothesis that *Wolbachia* factors can modify sperm cells prior to fertilization and suggest that further studies of the establishment of CI should focus on cells in the early stages of sperm development and in the cellular stages of chromosome remodelling.

**Data accessibility.** The accession number for the scRNA sequencing data reported in this paper is SRA: SAMN24018191, SAMN24018192. These data have been deposited in the Sequence Read Archive under BioProject: PRJNA788731. Data link: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA788731?reviewer=n7svq5k71ohhepvg14k41k4hhs>. 16S sequencing data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.stjq2c5w> [61]. R scripts used in this manuscript are available in electronic supplementary material, software file [62].

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