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. mel*) 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. mel* adult testes. Our findings show that *Wolbachia* affects many biological processes in *D. mel* 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.

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 mutualist 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 [25].

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. mel*), which provided a rich set of resources to further explore the functional basis of *Wolbachia-Drosophila* symbiosis [15]. 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 [16]. RNA-seq is also used to study *Wolbachia* and host gene expression profile in other species, such as nematodes and spider mites[17–19]. 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 [20], 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 [21]. 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 [22].

The process of spermatogenesis in *D. mel* 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 [23]. 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 [24]. *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 with 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 [25].

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 [22], 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 *w*Mel on the process of sperm maturation, based on an analysis of gene expression in the testes of *D. mel* adults with and without *Wolbachia* infection. *Wolbachia* strain *w*Mel has been widely used in the study of *Wolbachia*-host interactions [13, 28–30]. *w*Mel 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 w*Mel-infected and uninfected *D. mel* 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 w*Mel, hereafter referred to as DmelW, were kindly donated by Prof. Hu Haoyuan (Anhui Normal University). Five bacterial genes (*hcpA*, *fbpA*, *coxA*, *ftsZ*, and *gatB*) were amplified by PCR and sequenced. These sequences were submitted to the MLST website for *Wolbachia* strain identification, and we found that *D. mel* was indeed infected with *Wolbachia* strain *w*Mel (Additional file1). Uninfected *Wolbachia* line derived from the DmelW line, hereafter referred to as DmelT, were 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 7000Lux of light, 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. mel* [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 RNAhold 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 TansZol Up Plus RNA Kit (TransGen, Beijing, China) and sequenced by the BGISEQ-500 platform (BGI, Shenzhen, China) to obtain PE150bp paired-end transcriptome data in the size of 6 Gb. The data were submitted to NCBI under accession number PRJNA639180.

Differential gene expression calculations and enrichment analyses

We used Bowtie2 v2.2.5 for genomic reference alignment of the clean sequencing reads and RSEM to calculate the gene expression levels of each sample [34]. DEGseq 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/), 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)

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. mel* 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 didn't 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 TransScript One-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 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 $2^{-\Delta\Delta CT}$ method.

Data analysis

For the data analysis, a multiple t-test was performed in GraphPad Prism8 to analyze the significance of differences between both groups. A *p*-value assessed by Holm-Sidak method and *p*-value 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 2-fold 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 D-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 5-fold 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 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 2-fold 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).

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
	Drsl3	1.88	Defense response to fungus
	Takl1	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

Table 2
Main significantly enriched KEGG pathways associated with upregulated DEGs in DmelW testes.

	Gene symbol	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

	Gene symbol	log2 (DmelW/DmelT)	Q-value	KEGG pathway	Biological functions
	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
	spheroide	8.48	1.01E-70	Toll and IMD signal pathway	Defense response to gram-positive bacterium
	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

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 sterol transport were significantly upregulated in the testes of *Wolbachia*-infected *D. mel*. 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).

Table 3
Classification of lipid metabolism-associated genes that were differentially expressed in DmelW and DmelT testes, as identified by RNA-seq analyses.

Relative expression	Genes	Biological functions	KEGG pathway
Upregulated	CG18258, Yp1, Yp2, CG6296CG3699, CG6277, CG10116, CG15533, CG15534	Lipid catabolic process	NA
	Npc1b, Npc2f, Npc2e, Npc2d	Sterol transport; intracellular cholesterol transport	NA
	mag	Negative regulation of juvenile hormone biosynthetic process; lipase activity	NA
	CG11162	Lipid biosynthetic process	Steroid biosynthesis
	Acox57D-d	Lipid catabolic process	alpha-Linolenic acid metabolism
	Gba1a	Lipid catabolic process	Sphingolipid metabolism; Lysosome
	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

Notes: NA, no KEGG pathway was enriched.

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*, spheroide, *Takl1*, and *Drsl3*, as well as *PGRP-SC2*, which encodes a peptidoglycan recognition protein (Fig. 3c and 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 [20], 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. mel* 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. mel* 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. mel* and may be the cause of *Wolbachia*-induced CI [27, 39] were 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 sperm 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. mel*, identified by Darbyac *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 [17]. 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 DmelM 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 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].

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, CG10097, 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. mel* adult testis [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 testis 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 byproduct 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* not only induces 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. mel* 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. mel* 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.

Declarations

Authors' contributions Jinhua Xiao and Dawei Huang conceived the study. Weihao Dou analysed 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 softwares. All authors have read and approved the manuscript.

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Ethics approval Not applicable

Code availability Not applicable

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

Consent to participate Not applicable

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

Conflicts of interest The authors declare that they have no conflict of interests.

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Figures

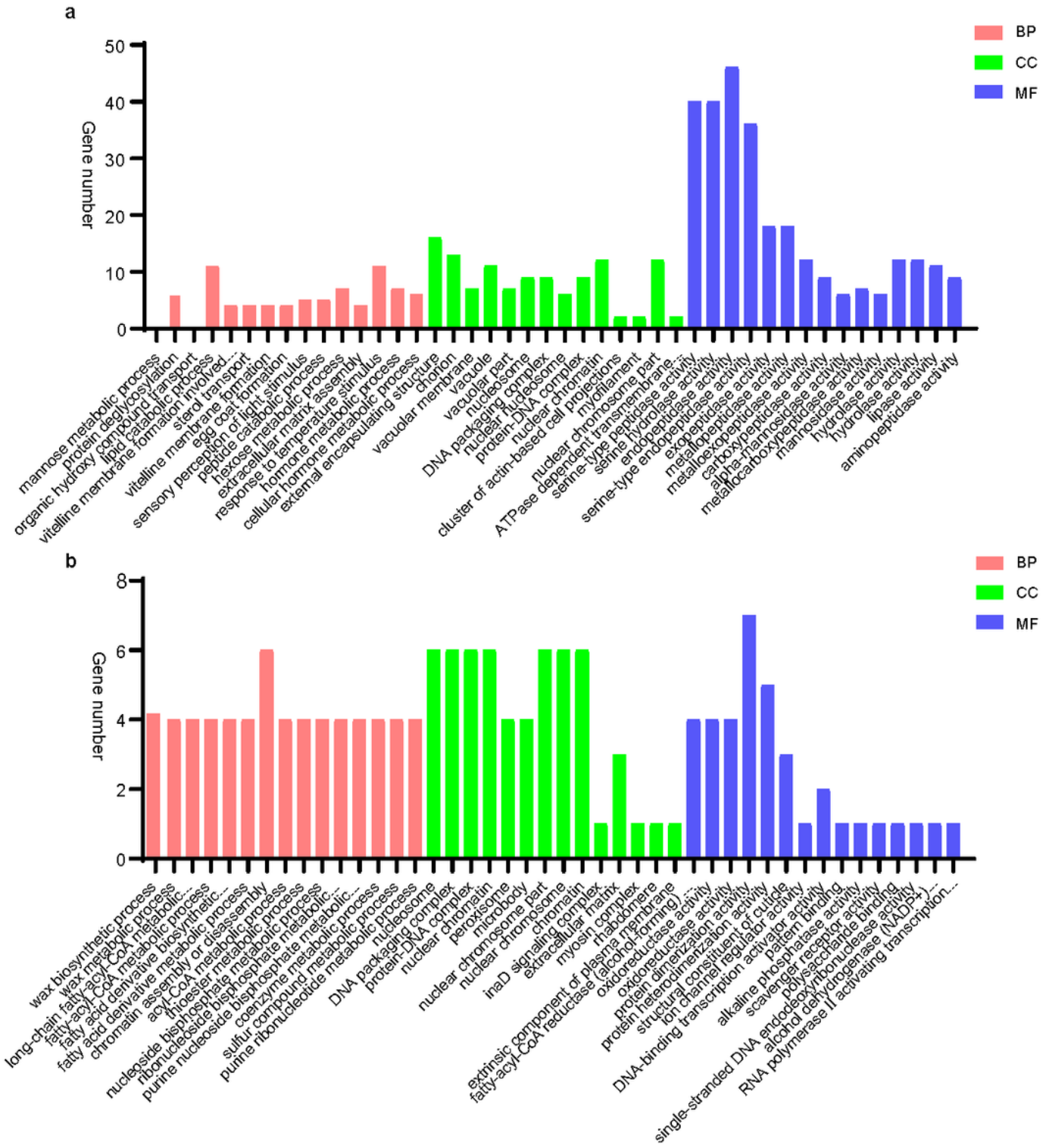

Figure 1

Top 15 terms from the functional GO enrichment analysis of upregulated (a) and downregulated genes (b) in the DmelW samples compared to DmelT samples. The horizontal axis represents the enriched items, and the vertical axis represents the gene number in each enriched item. BP, biological process; CC, cellular component, MF, molecular function.

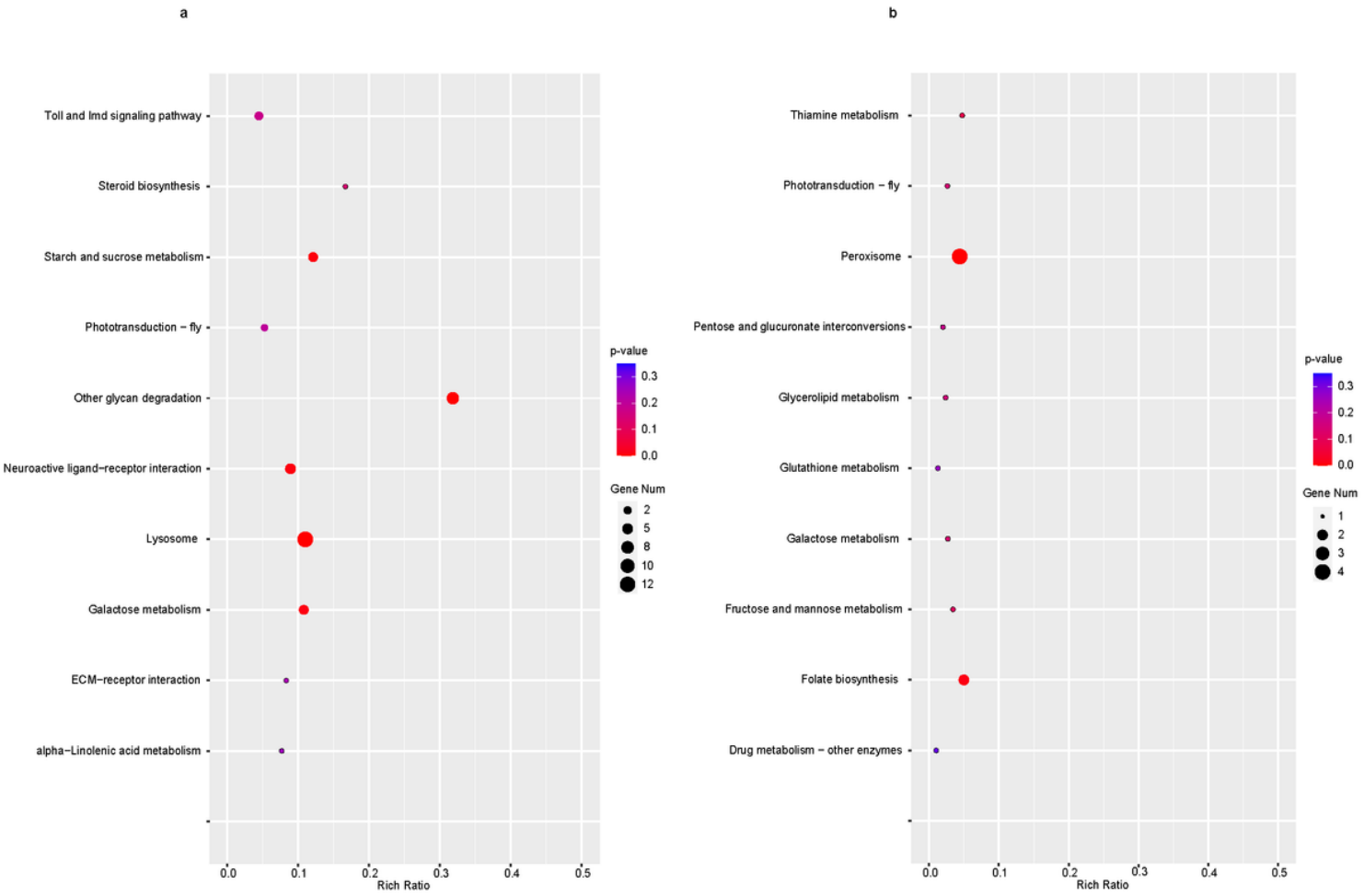

Figure 2

KEGG enrichment analysis of significant DEGs in the adult testes of DmelW and DmelT. Upregulated (a) and downregulated (b) genes in DmelW are shown.

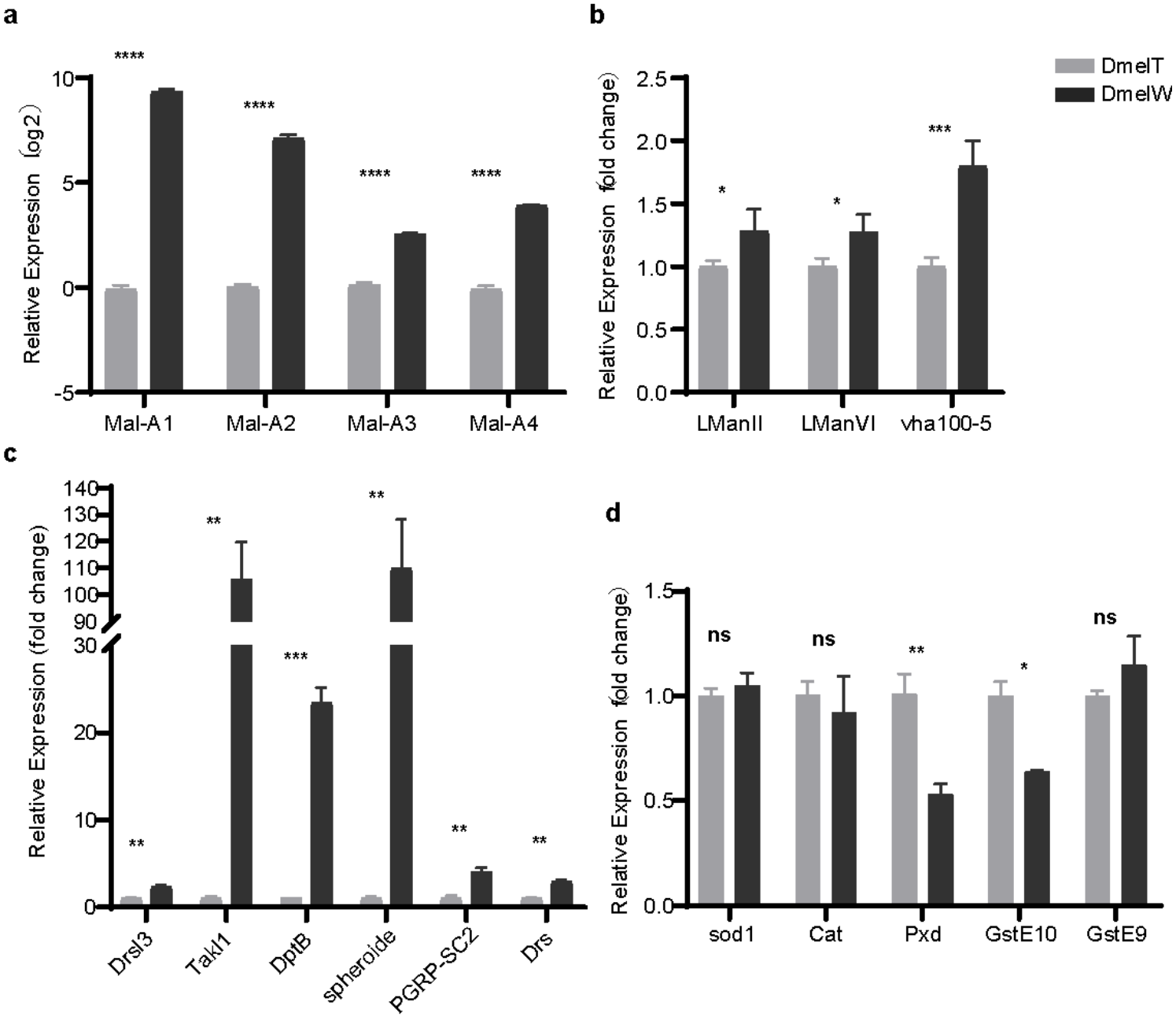

Figure 3

qRT-PCR validation of some of the significant DEGs between DmelW and DmelT testes in carbohydrate metabolism (a), lysosome (b), innate immune response (c), and ROS degradation (d); Statistical significance was determined using multiple t-test in Prism8. Bars indicate standard error. A p-value assessed by Holm-Sidak method. **: $p < 0.0021$, ***: $p < 0.0002$, ****: $p < 0.0001$, $n = 3$.

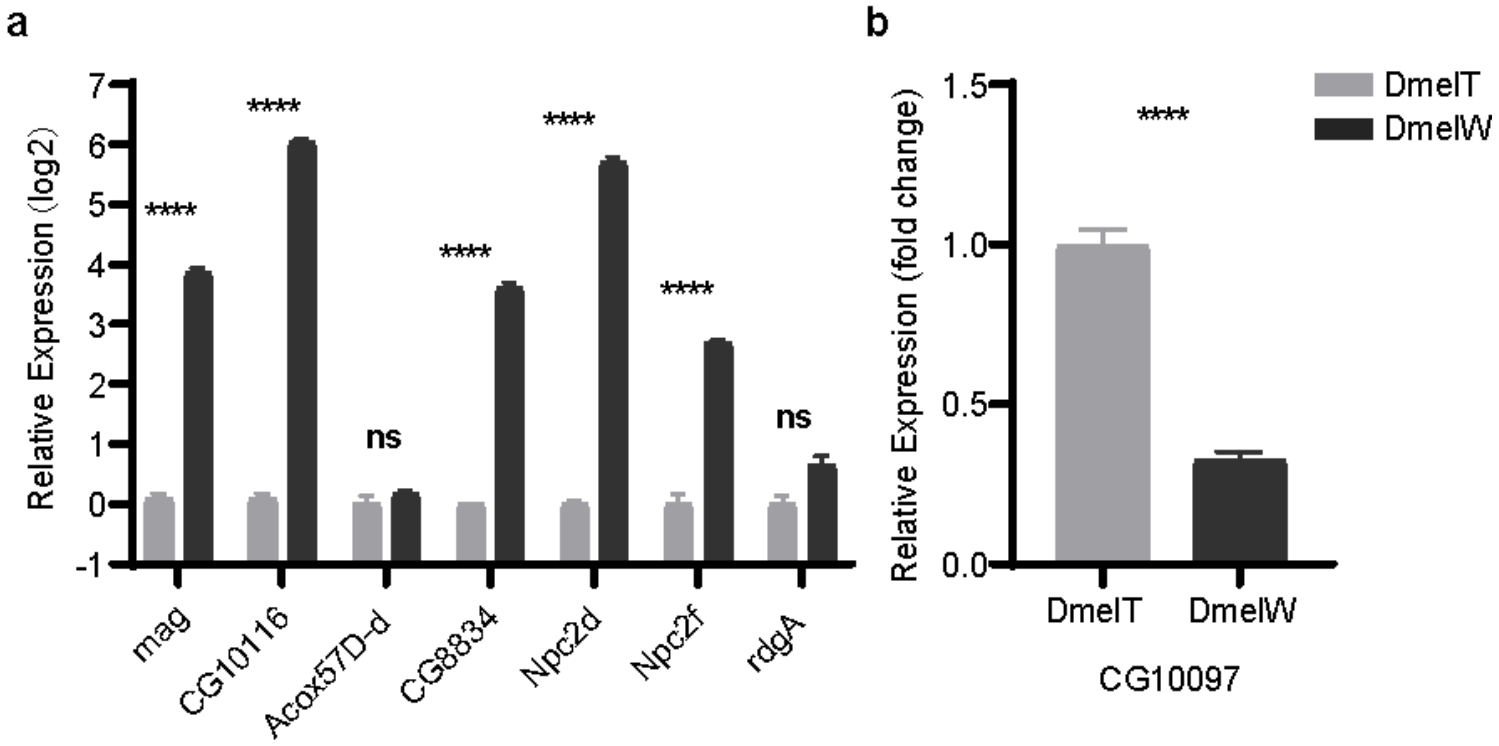

Figure 4

qRT-PCR validation of lipid metabolism-related DEGs. Bars indicate standard error. A p-value assessed by Holm-Sidak method. ****: $p < 0.0001$, n = 3, ns: not significant, n = 3.

Supplementary Files

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- gatB.ab1
- hcpA.ab1