The Association of Wolbachia on the Gene Expression in Drosophila Adult Testis

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Research article

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Abstract

Background: *Wolbachia* is a type of intracellular symbiotic bacteria widely distributed in arthropods including most insects and nematodes. These maternally inherited bacteria can regulate the host's reproductive system in various ways for their own vertical transmission. Since the identification of *Wolbachia* in many insects, the relationship between *Wolbachia* and host has attracted great interest. *Wolbachia* must rely on the host cells to survive, and they can also improve the fitness of the host through a variety of ways. However, the molecular basis of interaction between *Wolbachia* and their host has not been well resolved so far.

Results: We performed transcriptome sequencing on testis tissues of adults of *Wolbachia*-infected and *Wolbachia*-free *Drosophila melanogaster*. Comparison of gene expression profiles revealed 471 significantly differentially expressed genes that involved in cell metabolism, cell membrane component correlation and hydrolysis process.

Conclusions: Our results show that lipid and carbohydrate metabolism are more active in *Wolbachia*-infected testis than in *Wolbachia* free testis. This work strengthens our general understanding of the *Wolbachia*-host intracellular relationship and may provide a new perspective for *Wolbachia*-mediated virus-blocking.

Background

Wolbachia is a class of intracellular bacteria that are widely spread in arthropods and nematodes, and this maternally transmitted bacteria can regulate the host's reproductive system in a variety of ways to increase its chances of transmission, such as cytoplasmic incompatibility (CI), parthenogenesis inducing (PI), male killing (MK), femalization and enhancement of female fertility [1–4],and these manipulations are considered as a kind of “selfish strategies” to increase their infection rate in the host population and reduce host fitness [1, 5]. But in other ways *Wolbachia* can significantly improve host fitness. For example, *Wolbachia* is necessary for nematode growth and development, as the adult worms were retarded, ovaries degenerated, and embryos could not form when *Wolbachia* was remove from nematodes by antibiotics [6]. In insect hosts, *Wolbachia* can increase the host’s fertility [7–11], and enhance the insect host’s resistance to many pathogens (such as RNA virus) [12–14]. *Wolbachia* can also provide vitamins to the host and give the host a clear growth advantage in the face of adversity [1, 15]. In addition, the host longevity, olfactory response, immunity and stem cell proliferation were also affected by *Wolbachia* [16–18]. It is expected that *Wolbachia* have evolved mutualistic relationship with many of their hosts [19] .

Wolbachia infect a remarkable range of insect hosts suggests their interaction with various host metabolic pathways for their successful intracellular maintenance within a host [20]. This interactive relationship is complex and has received extensive attention. Recent transcriptomic, genomic, and proteomic data have revealed that *Wolbachia* affects many physiological processes in the host. Previous

studies that combined genome-wide RNAi screening and new high-throughput fluorescence in situ hybridization technology in *Drosophila* cell lines have found that a large number of host genes may be involved in the alteration of *Wolbachia* level. These genes are involved in lipid metabolism, transport, protein degradation, translation, and cell cycle [21, 22], indicating that many host's metabolic pathways have an obvious impact on the survival of *Wolbachia*. A genome-wide analysis of various insect-related *Wolbachia* strains has indicated that *Wolbachia* has a complete and conserved glycolysis pathway and riboflavin (vitamin B2) synthesis pathway [23]. Riboflavin synthesized by *Wolbachia* has a significant contribution to the growth and development of bed bugs [15]. However, *Wolbachia* is highly dependent on the host carbon source (such as phosphoglyceride), amino acids (such as alanine) and phospholipids [23]; *Wolbachia* lacks lipid metabolism genes, especially lipid A synthesis genes [24], so *Wolbachia* must obtaining these lipids from the host to support cell growth. In addition, the proliferation of *Wolbachia* in the host is cholesterol-dependent, and *Wolbachia* lacks cholesterol synthesis genes, so their survival is likely related to the transport of host cholesterol. A recent study also showed that *Wolbachia* can be localized in the endoplasmic reticulum, which is the main site of lipid metabolism, affecting the distribution of the endoplasmic reticulum and obtaining vacuole membranes from it [25]. Therefore, the above studies have indicated that *Wolbachia* has a close nutritional symbiosis relationship with the host, so lipids and carbohydrate metabolism is likely to play an extremely important role in the symbiotic relationship between *Wolbachia* and the host. However, the interaction between *Wolbachia* and the host in lipid and carbohydrate metabolism has not been studied in details.

Wolbachia is mainly localized in the reproductive tissues of host [26], which is a good material for studying the interaction between *Wolbachia* and host. Previous studies have showed that *Wolbachia* affects the early spermatogenesis process [27]. The gene expression pattern between the *Drosophila melanogaster* larval testis in the *Wolbachia*-infected and the *Wolbachia*-free samples observed differential expression of genes related to metabolism, immunity, reproduction and other functions, some of which that may be related to spermatogenesis, including *Ance*, *Iola* and *Mst84Db*, were down-regulated in *Wolbachia*-infected sample, inducing abnormal spermatogenesis that causes *Wolbachia* to "modify" sperm, which may be the cause of CI. However, in *Drosophila*, mature sperms are continuously produced in the testis after eclosion but not in the larval stage [28]. Therefore, the purpose of this study is to explore the putative effect of *Wolbachia* in the process of sperm maturation, based on the gene expression in the testis tissue of *Drosophila* adults.

We selected *Wolbachia*-infected and *Wolbachia*-free *Drosophila* adult testis tissues for RNA-seq, and analyzed the differentially expressed genes. A total of 472 genes are different expressed at least a 2 fold change (q-value < 0.1%) between *Wolbachia*-infected and *Wolbachia*-free testis, involving a variety of physiological processes such as innate immune immunity, carbohydrate metabolism, and lipid metabolism. Most of the differentially expressed genes involved in innate immunity including Toll and IMD pathway were up-regulated in the presence of Wolbachia. More interestingly, lipid and carbohydrate metabolism related gene were significant up-regulated in the presence of *Wolbachia*, while epidermal wax ester synthesis related genes were down-regulated by *Wolbachia*. Our research found for the first time that the presence of *Wolbachia* is associated with more active lipid metabolism and carbohydrate

metabolism in the host. These results can provide important evidence for elucidating the mechanism of *Wolbachia*-host intracellular relationship.

Result

Transcriptome sequencing data of Drosophila adult testis

We performed transcriptome sequencing on testis tissues of adults (one day after eclosion) of *Wolbachia*-infected (WInM) and *Wolbachia*-free (WUnM) *Drosophila melanogaster*. Comparison of gene expression profiles revealed 471 significantly differentially expressed genes (at least 2fold, q-value < 0.1%), with 402 genes up-regulated and 69 genes down-regulated in the presence of *Wolbachia* infection.

The significantly differentially expressed genes between Wolbachia-infected (WInM) and Wolbachia-free (WUnM) samples are involved in many metabolic pathways

We performed Gene Ontology classification analysis on the 472 significantly differentially expressed genes and found that they were involved in cell metabolism, cell membrane component correlation and hydrolysis process (Fig. 1a, b). Results of KEGG enrichment analysis showed that up-regulated genes in WInM were mostly involved in transport, signal transduction, immune response, glucose metabolism, lipid metabolism and digestion processes, while down-regulated genes in WInM was involved in the insect epidermal wax ester synthesis pathway (Fig. 2).

We detected altogether six significantly up-regulated genes in WInM were involved in the innate immune pathway of the *Drosophila* (Table 1 & Fig. 3a), all of which including *DptB*, CG9673, *spheroide*, *Takl1*, *Drsl3*, and the peptidoglycan recognition protein gene *PGRP-SC2* were functional in Toll and IMD pathways.

Table 1
Genes related to immune response that are differentially expressed (≥ 2 fold changes, q-value < 0.1%) in testes of *Wolbachia*-infected flies compared to *Wolbachia*-free flies.

Gene Symbol	log2 (WInM/WUnM)	KEGG Pathway	Biological Process
DptB	1.41	Toll and Imd signaling pathway	defense response to Gram-negative bacterium
CG9673	8.09	Toll and Imd signaling pathway	proteolysis
spheroide	8.48	Toll and Imd signaling pathway	positive regulation of Toll signaling pathway,
			defense response to Gram-positive bacterium
Takl1	2.02	Toll and Imd signaling pathway	defense response to fungus
Drsl3	1.88	Toll and Imd signaling pathway	phosphorylation,activation of MAPKK activity
PGRP-SC2	1.54		negative regulation of innate immune response

Genes related to carbohydrate metabolism, especially the genes related to the hydrolysis of sucrose and starch, including *Mal-A1*, *Mal-A3* and *Mal-A4*, were significantly up-regulated in *Wolbachia*-infected *Drosopshila* testis. Among them, *Mal-A1* was up-regulated in *Wolbachia* infected testis by nearly 700 folds (Fig. 3b). In addition, the expression of UDP-glycosyltransferase genes such as CG5724, CG5999, *Ugt86Dh* were also significantly up-regulated in *Wolbachia*-infected testis (Fig. 3d), and these genes are necessary for *Wolbachia* in the synthesis of LipidA and further synthesis of lipopolysaccharide (LPS) [29].

We also found that the several genes involved in lipid metabolism were significantly up-regulated in *Wolbachia*-infected *Drosophila* adults testis, among which the genes of mag and CG10116 were involved in lipid degradation, genes of CG5804 and CG8628 encoded acetyl-CoA binding proteins, gene of CG8834 was related in fatty acid synthesis, gene of *Acox57D-d* was functional in fatty acid degradation, and genes of *Npc2f* and *Npc2d* were involved in intracellular cholesterol transportation (Table 2 & Fig. 3c). In contrast, the genes of CG10097, CG1441, CG13091, and CG17560, which were involved in the synthesis of other lipid derivatives such as fatty alcohols from fatty acid, were significantly down-regulated in *Wolbachia*-infected testis, which were annotated to have fatty acyl-CoA reductase (alcohol-forming) activity, or be involved in the biosynthesis of insect cutin and wax (Table 2).

Table 2
Classification of genes related to Lipid metabolism that are differentially expressed (≥ 2 fold changes, q-value < 0.1%) in testes of *Wolbachia*-infected flies compared to uninfected flies .

	Gene Symbol	log2 (WInM/WUnM)	GO analysis	KEGG pathway
Lipid metabolism	CG5804	9.67	fatty-acyl-CoA binding	
	CG8628	4.29	fatty-acyl-CoA binding	
	CG10116	6.63	lipid catabolic process	
	mag	5.84	lipid metabolic process	
	Npc2f	1.33	intracellular cholesterol transport	
	Npc2d	4.33	intracellular cholesterol transport	
	Acox57D-d	1.66	fatty acid beta-oxidation	Fatty acid degradation
	CG8834	3.08	fatty acid biosynthetic process	
	CG9084	3.53	phospholipid scramblase activity	
	CG1441	-1.08	alcohol-forming fatty acyl-CoA reductase activity	Cutin and wax biosynthesis
	CG13091	-1.16	alcohol-forming fatty acyl-CoA reductase activity	Cutin and wax biosynthesis
	CG10097	-1.30	alcohol-forming fatty acyl-CoA reductase activity	Cutin and wax biosynthesis
	CG17560	-1.24	alcohol-forming fatty acyl-CoA reductase activity	Cutin and wax biosynthesis

Discussion

Wolbachia and its host have a long history of co-evolution, and the interaction between both parters is very complex, which has not been clearly clarified so far [30]. *Wolbachia* is mainly located in the insect host reproductive system [26], including female ovary and male testis which makes them good materials for studying *Wolbachia*-host interaction. Previous results based on the transcriptomic data of the testis

tissue of the third instar larvae of *Drosophila* have shown that *Wolbachia* infection can affect the expression of genes related to spermatogenesis and thus may induce CI [27]. In this study, we focused on the gene expression in the adult testes of the *Wolbachia*-infected and *Wolbachia*-free *Drosophila melanogaster* to investigate the possible effects of *Wolbachia* on the host reproductive system during the process of sperm maturity. The results showed that the expression of genes involved in innate immune system and multiple metabolic pathways, especially lipid and carbohydrate metabolism, were significantly different between *Wolbachia*-infected and *Wolbachia*-free *Drosophila* adult testis. We speculate that *Wolbachia* may competes with its host for carbohydrate and lipid metabolism resources, on the other hand, *Wolbachia* also provides vitamin for the host (Fig. 4).

Wolbachia **is associated with the high expression of innate immune genes in native host**

When insects are infected by pathogenic bacteria, the host innate immune responses such as Toll and IMD signaling pathways are activated and then produce a variety of antibacterial peptides (AMPs) [31]. *Wolbachia* can survive in host cell, and some data have shown that it does not induce immune responses in its native host [12, 32–34]. However, our results showed that *Wolbachia* was related in enhanced immune responses in its native host testis, including multiple genes in the Toll and IMD pathways, such as *DptB*, CG9673, *spheroide*, *Takl1* and *Drsl3*. For example, the protein encoded by the *DptB* gene is an antimicrobial peptide induced by the IMD signal pathway, which is specifically produced in insect fat bodies and can resist gram-negative bacteria infection [35], indicating that *Wolbachia*, as a gram-negative bacteria, can still induce the immune response in its native host. In addition, *spheroide* and *Takl1* are usually involved in activating Toll signaling pathways in resisting gram-positive bacteria and fungal infections [36]. It can be seen that in the naturally infected host, *Wolbachia* may still lead to an increase in its immune response. The enhanced host's immune system may be a "double-edged sword" for *Wolbachia*, which is harmful to itself and limits infection with other pathogenic bacteria, prevents the pathogenic bacteria for snatching intracellular resources. Of course, *Wolbachia* may also escape the host ’s immune system in various ways. The expression level of peptidoglycan recognition protein *PGRP-SC2* is significantly higher in Wolbachia infected testis, *PGRP-SC2* can negatively regulate the IMD signaling pathway by hydrolyzing peptidoglycan, preventing the activation of the constitutive IMD pathway, thereby maintaining the balance between immune tolerance and immune response for *Wolbachia* infection [37]. Our subsequent transcriptome data (unpublished) shows that the expression levels of Toll and IMD pathway related genes are not different between infected and uninfected *Drosophila* female ovaries, so it can be speculated that the relationship between *Wolbachia* and male or female hosts is different. *Wolbachia* may escape the host's immune system in other ways in females, for example, some studies have pointed out that *Wolbachia* itself can encode Peptidoglycan Amidase to avoid the host's immune system [38].

Wolbachia **is associated with the more active carbohydrate metabolism process in the host**

In this study, we noticed that the carbohydrate metabolism was more active in the *Wolbachia*-infected sample, which might be associated with the infection of *Wolbachia*. First, the expression levels of *Mal-A1*,

Mal-A3, and *Mal-A4* genes related to starch and sucrose hydrolase activity were significantly up-regulated in *Wolbachia* infected *Drosophila* testis. These genes can accelerate the formation of D-glucose, which is the initial substrate of glycolysis. It has been reported that insect associated *Wolbachia* exhibits a complete glycolysis metabolic pathway [23], so it is possible that *Wolbachia* compete with the host to consume the glycolysis substrate-glucose, resulting in a more active sugar metabolism in the host.

Second, the expression levels of UDP-glycosyltransferase genes including CG5724, CG5999, and *Ugt86Dh* were significantly up-regulated in *Wolbachia*-infected testes. Actually, there is no gene involved in UDP-glycosylation in the *Wolbachia* genome [23], even though *Wolbachia* need this function. *Wolbachia* must synthesize its own cell wall and Lipopolysaccharides (LPS) is an essential component of the cell wall [11]. The glycosylation reaction is a very important step in the biosynthesis of LPS, in which the host glycosyltransferase plays vital function for *Wolbchia* to survive [39]. Our results that the glycosyltransferase genes were up-regulated in the *Wolbachia*-infected host indicated that *Wolbachia* might heavily rely on the host in the process of LPS synthesis.

Wolbachia **is associated with the more active lipid metabolism in the host**

We detected significantly differentially expressed genes in lipid metabolism between *Wolbachia*-infected and *Wolbachia*-uninfected *Drosophila* testes. Two genes including CG5804 and CG8628 were significantly up-regulated in *Wolbachia*-infected *Drosophila* testis, both of which belonged to the acetyl-CoA binding protein (ACBP) family which were involved in regulating the expression of genes related to lipid metabolism. Interestingly, we detected that lipolysis-related genes such as *mag* and CG10116 were also up-regulated in *Wolbachia*-infected *Drosophila* testis, and this genes can regulate the storage of triacylglycerol (TAG) and maintain the balance of fat metabolism [40, 41]. Finally, the expression levels of genes *Npc2f* and *Npc2d* involved in intracellular cholesterol transport were also significantly up-regulated in *Wolbachia*-infected testis. All of these results indicated that the existence of *Wolbachia* was related to the high expression of genes related to lipid metabolism and transport in the host, suggesting that it may induce a more active fat metabolism in the host.

On the contrary, a gene (CG10097) in lipid derivatives synthesis was significantly down-regulated in *Wolbachia*-infected *Drosophila* testis tissue. Both GO molecular functional analysis and KEGG analysis showed that this gene encoded fatty acyl-CoA reductase (alcohol-forming), which was the key enzyme in the biosynthesis of insect epidermal wax esters or insect cutin and wax. This indicated that during the period of proliferation of *Wolbachia* in the host, the host might concentrate fatty acid resources for necessary lipid metabolism for survival, which thus restricted its conversion process to other lipid derivatives.

Conclusions

Wolbachia and the insect host has had a long co-evolutionary history that formed a mutually beneficial symbiosis. *Wolbachia* provides the necessary nutrients for the host, and its survival is strictly dependent on the host. Our RNA-seq data based on *Drosophila* adult testis indicated that *Wolbachia* may affect

various physiological pathways of the host, such as immunity, glucose metabolism and lipid metabolism. These data provide important molecular evidence for the *Wolbachia*-host intracellular relationship. Subsequent analysis of transcriptome data in *Drosophila* ovaries may help further understand the differences in *Wolbachia*-host molecular interactions between male and female hosts.

Methods

Fruit fly rearing

Drosophila melanogaster in this laboratory was kindly donated by Prof. Hu Haoyuan in Anhui Normal University. The standard medium of *Drosophila* corn flour was used for feeding. 60 g corn flour, 30 g brown sugar, 5 g sucrose, 1 g sodium benzoate, 6 g agar powder, 800 ml water was added and boiled for 5 min. After cooling, 5 g yeast powder was added to make fruit fly medium. The fruit flies were reared in an artificial climate box (Ningbo Jiangnan Instrument Factory), light: dark = 14L: 10D, relative humidity 40%, light 7000Lux [42].

Wolbachia **-free fruit fly strains**

We used the MLST method to detect the *Wolbachia* strain in fruit flies [43] and found that the *Drosophila melanogaster* naturally infected the *Wolbachia* wMel strain, and the *Wolbachia*-infected *Drosophila melanogaster* strain was named WIn. We prepared tetracycline stock solution 10 mg/mL, adding 2 mL tetracycline stock solution per 100 g corn flour medium [44]. After continuous treatment for three generations, by PCR detection of *Wolbachia* wsp, ftsz, and 16 s genes, we obtained *Wolbachia*-free fruit fly strain WUn and then transferred them to standard medium and continuously cultivated for more than 5 generations to remove the effects of antibiotics.

RNA-seq

The WIn and WUn male fruit flies that had been reared in corn flour medium for one day after eclosion then dissected in RNase-free water, and the complete testis was placed in RNAhold and stored at -80 °C. Total RNA was extracted by using *TansZol* Up Plus RNA Kit, about 10 *Drosophila* testes were used for each sample. Using PE150bp pair-end transcriptome sequencing in BGISEQ-500 platform (BGI, Shenzhen, China), the amount of sequencing data requires 6G for each sample. The sequnces were submitted to NCBI with the accession number of PRJNA639180.

Quantitative real-time PCR (qPCR)

To further investigate the differentially expressed genes identified by RNA-seq, 17 genes were selected for qRT-PCR analysis. Specific primers for the 17 genes and RP49 (Ribosome protein 49 reference gene) were designed by NCBI primer-BLAST (Additional file 1).

RNA extraction from WIn and WUn testis was carried out using TransZol Up Plus RNA Kit (TransGen, Beijing, China) according to the instructions, RNA reverse transcription was performed using Transscript

One-Step gDNA Renover and cDNA Synthsis SuperMix (TransGen, Beijing, China), 1 μg RNA was used for reverse transcription, gDNA remover 1μL, Oligo(dT) 1μL, 2xTS Reaction Mix 10μL, *TransScript* RT/RI Enzyme Mix 1μL, added Nuclease-free water for total volume 20μL. Reaction conditions : 42℃ for 30 min, then 85℃ for 5sec. QPCR verifies differential gene expression. The primers were shown in Table S1. We used the ΔΔCt method, by using the PerfectStart Green qPCR SuperMix Kit (TransGen, Beijing, China), the reaction system is cDNA 1μL, Forward/Reverse Primer each 0.4μL (10 μM/L), 2xPerfectStart Green qPCR SuperMix 10μL, and Nuclease-free water 8.2μL. Reaction conditions: Pre-reaction at 94 ° C for 30 s, then 40 cycles of 94 ° C for 5 s, 60 ° C for 30 s, and then the dissolution step was performed.

Data analysis

For data analysis, multiple t test in Graphpad prism8 was used to analyze the significance of differences between two groups. *p*-value of less than 0.05 was considered significant.

Abbreviations

WInM
Wolbachia-infected male; WUnM: *Wolbachia*-uninfected male; WIn: *Wolbachia*-infected *Drosophila melanogaster* strain; WUn: *Wolbachia*-uninfected *Drosophila melanogaster* strain.
LPS:Lipopolysaccharides; ACBP:Acetyl-CoA binding protein family; TAG:Triacylglycerol; DHAP:Dihydroxyacetone phosphate.

Declarations

Acknowlegements

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Authors' contributions

JHX and DWH conceived the study. WHD analysed the data, performed QPCR verification and wrote the paper. All authors have read and approved the manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Figures

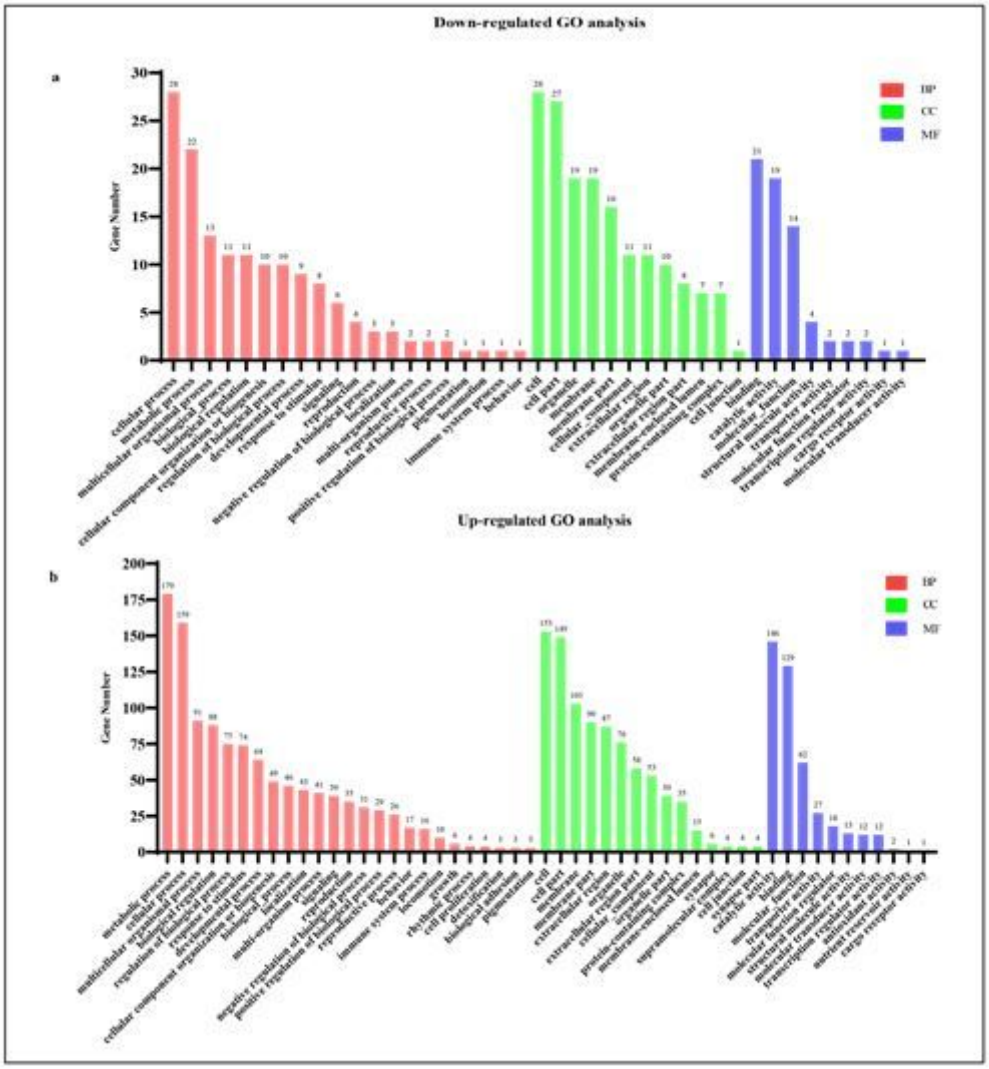

Figure 1

Functional GO enrichment analysis of the down-regulated genes(a) or up-regulated genes(b) in Wolbachia-infected Drosophila melanogaster adult testis compared to Wolbachia-free sample.

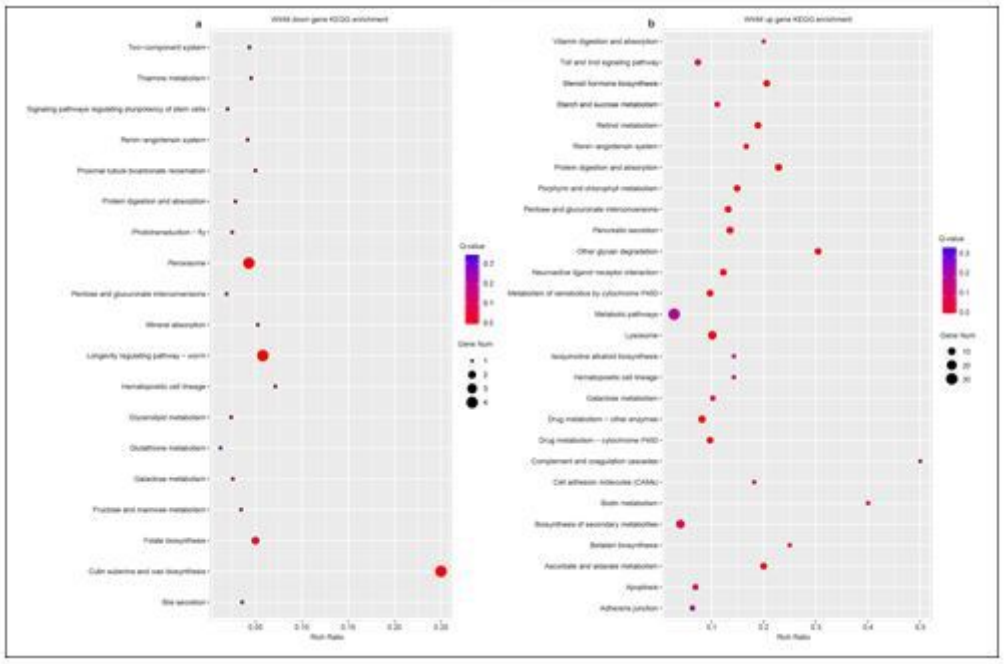

Figure 2

KEGG enrichment analysis on the significantly differentially expressed genes between the adult testis of Wolbachia-infected (WInM) and Wolbachia-free (WUnM) Drosophila melanogaster. The results for genes down-regulated in WInM is shown in (a) and up-regulated genes in WInM is shown in (b).

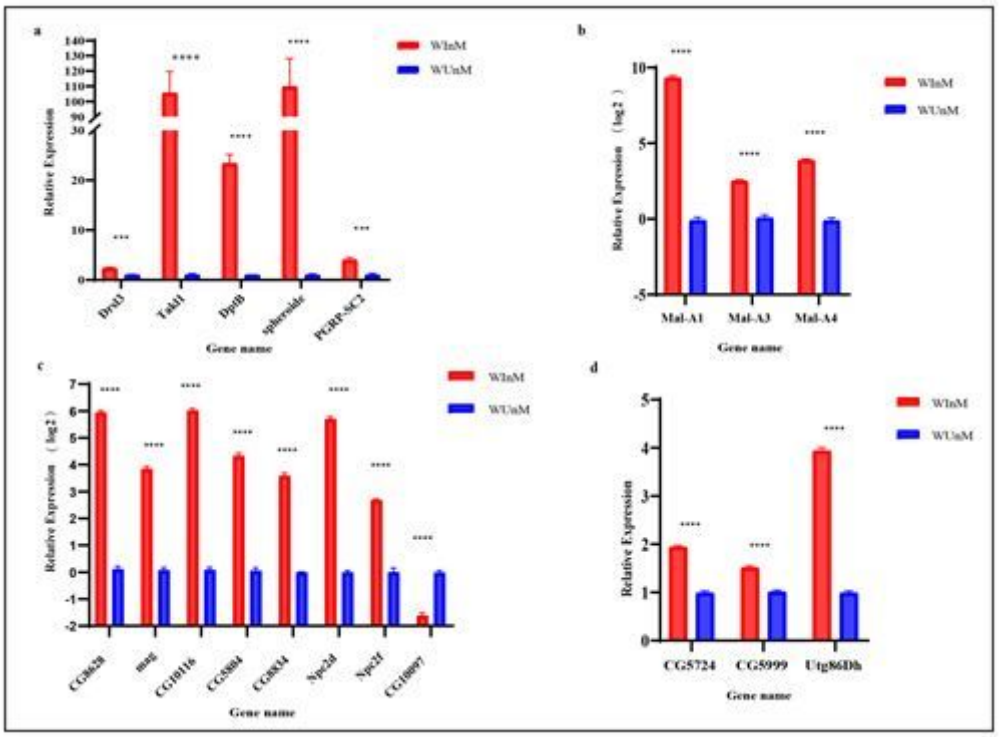

Figure 3

Quantitative PCR validation on some of the significantly different expressed genes between Wolbachia-infected and Wolbachia-uninfected Drosophila testis in innate immune response(a); carbohydrate

metabolism(b,d) and lipid metabolism(c). Statistical signifificance was determined with multiple t-test in Prism8, “***” and “****” indicate significant differences with p-value<0.001and p-value<0.0001, respectively, n=3.

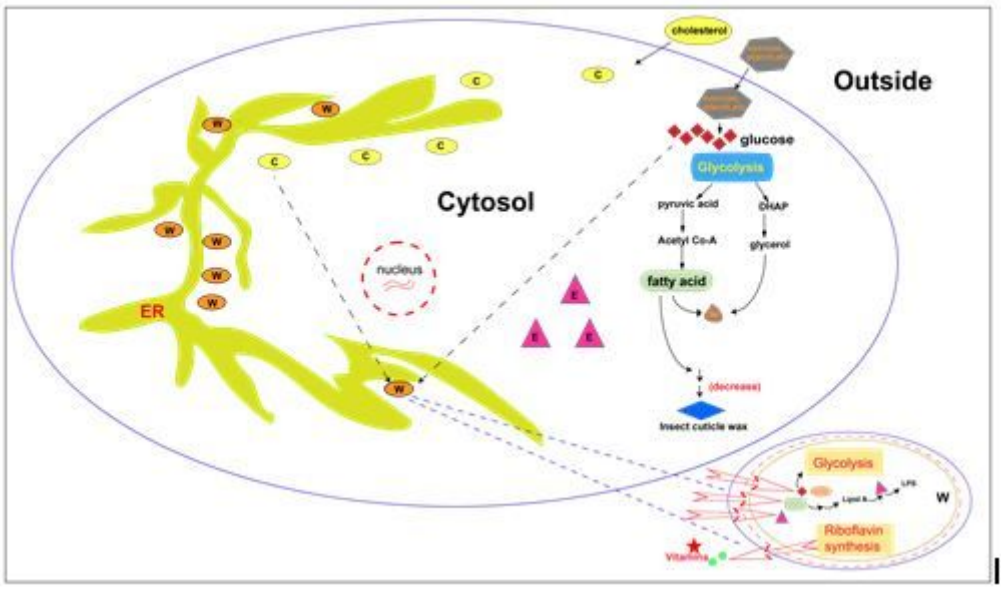

Figure 4

Schematic diagram of the possible metabolic interactions between Wolbachia and host. By the picture, we want to show how host cells absorb cholesterol (Labeled as C in yellow ovals), starch, surose and other nutrients from the outside; Starch and sucrose are hydrolyzed to glucose (red diamond), which then participates in glycolysis to generate fatty acids and other substances. On the other side, Wolbachia (Labeled as W in orange ovals), mainly located in Endoplasmic reticulum (ER), plunder the glucose of the host, resulting in the acceleration of hosts’ glucose metabolism, but inhibition of the synthesis of insect cuticle wax (blue diamond) with fatty acids; Meanwhile, Wolbachia also obtain cholesterol, fatty acids and other substances from the host. The plundered glucose is used for the glycolysis of Wolbachia, and fatty acids are used to synthesize LPS under the action of the host glycosyltransferase (Labeled as E in pink triangle). In return, Wolbachia provides vitamins for the host (Marked with a red star) [23]. The solid lines with arrow indicate the biological reaction process, the dotted lines with arrow indicate that Wolbachia compete with host for some metabolic resources, and the hollow arrows indicate the processes of material exchange among extracellular environment, the host cell, and Wolbachia. DHAP: Dihydroxyacetone phosphate.

Supplementary Files

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