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The Intruding *Wolbachia* Strain from the Moth Fails to Establish Itself in the Fruit Fly Due to Immune and Exclusion Reactions

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Abstract

Wolbachia is capable of regulating host reproduction, and thus of great significance in preventing the spread of insect-borne diseases and controlling pest insects. The fruit fly *Drosophila melanogaster* is an excellent model insect for understanding *Wolbachia*-host interactions. Here we artificially transferred the *w*Ccep strain from the rice moth *Corcyra cephalonica* into *D. melanogaster* by microinjection. Crossing experiments indicated that *w*Ccep could induce a high level of CI in the phylogenetically distant host *D. melanogaster* and imposed no negative fitness costs on host development and fecundity. Based on quantitative analysis, the titres of *w*Ccep and the native *w*Mel strain were negatively correlated, and *w*Ccep could only be transmitted in the novel host for several generations (G_0 to G_4) after transinfection. Transcriptome sequencing indicated that the invading *w*Ccep strain induced a significant immune- and stress-related response from the host. An association analysis between the expression of immune genes *attacin*-D/*edin* and the titre of *Wolbachia* by linear regression displayed a negative correlation between them. Our study suggest that the intrusion of *w*Ccep elicited a robust immune response from the host and incurred a competitive exclusion from the native *Wolbachia* strain, which resulted in the failure of its establishment in *D. melanogaster*.

Abbreviations

CI	Cytoplasmic incompatibility
AMPs	Antimicrobial peptides
qPCR	Real-time quantitative polymerase chain reaction
GAPDH	Glyceraldehyde phosphate dehydrogenase
Ct	Cycle threshold
WT	Wildtype
IN	Infected
UN	Uninfected
hpi	Hours post-injection.
bp	Base pairs
Kb	Kilobase
DEG	Differentially expressed genes
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOBAS	KEGG orthology-based annotation system
FPKM	Fragments per kilobase of transcript per million mapped reads
RNA-Seq	RNA sequencing (whole transcriptome shotgun sequencing)
SNK	Student Newman Keuls

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Introduction

Arthropods harbor a variety of microorganisms, and *Wolbachia* are perhaps among the most commonly occurring facultative bacterial endosymbionts [1]. This group of vertically transmitted Gram-negative bacteria attracted more and more attention for their capability of manipulating host reproduction by causing cytoplasmic incompatibility (CI), feminization, male killing, and parthenogenesis induction

[2], which is of great significance in pest control and human disease transmission [3].

The fruit fly *Drosophila melanogaster* uses multiple innate defense strategies to combat bacterial infection, many of which are also used by higher organisms including human beings [4]. These defense strategies include physical barriers and immunity: local immune response in the barrier epithelia by producing antimicrobial peptides (AMPs) and reactive oxygen species, cellular immunity via phagocytosis and encapsulation, and humoral immunity by synthesizing AMPs in the fat body. The signaling pathways regulating the production of AMPs were identified using the *Drosophila* model [5]. Seven groups of AMPs were characterized, among which Diptericin, Drosocin, and Attacin are highly effective against Gram-negative bacteria [6, 7]. On the other hand, the cellular encapsulation is a dramatic defense response mediated by lamellocytes in *Drosophila* [8]. Edin (elevated during infection) acted as an important determinant of the encapsulation response in *D. melanogaster* larvae [9]. In the past decades, remarkable progresses have been made in insect immunity, although the mechanisms underlying the insect-*Wolbachia* interactions are only partially understood [10]. *Wolbachia* are very common in *Drosophila* but they cannot be cultured outside of host cells [11]. Recognition of bacteria by *Drosophila* is achieved through the sensing of specific forms of peptidoglycan by peptidoglycan recognition proteins (PGRPs). The discovery of PGRP-LE as an intracellular sensor of Gram-negative bacteria may be among the important advances in understanding the immune defense of insects to *Wolbachia* [12]. It was reported that the PG-associated lipoprotein (PAL) was located on the cell membrane of *Wolbachia* [13]. PAL was known to specifically bind diaminopimelic acid (DAP) [14]. Therefore, *Wolbachia* can be recognized by PGRPs which then trigger the Imd pathway and subsequent AMP generation [15]. Nevertheless, up to now, the molecular mechanism of insect-*Wolbachia* and how the titer is controlled in vivo is poorly understood, particularly when multiple infections occur.

The success of *Wolbachia* is attributed to efficient maternal transmission and manipulations of host reproduction commonly through CI [16]. CI is affected by both host and *Wolbachia* [17, 18]. For instance, CI factor A (CifA) encoded by syntenic loci within *Wolbachia*'s WO prophage region played a key role in the rescue of CI [19], which was further supported by a recent study using two conspecific *Wolbachia* strains from *Drosophila pandora* [20]. Moreover, the strength of CI was correlated with the density or titre of *Wolbachia* [21], which appeared to be influenced by both host- and *Wolbachia*-intrinsic factors [22, 23]. It can be expected that the titre of *Wolbachia* should reflect a balanced interaction between host defense (immunity, resistance and tolerance) and *Wolbachia* anti-defense. Previous studies showed that the native *Wolbachia* strain did not elicit an AMP-based immune response in the host, while a strong induction of AMP gene expression was observed when *Wolbachia* were introduced into novel hosts [24–26]. Nevertheless, the mechanisms underlying the complex interactions between host insects and co-existing *Wolbachia* strains are still unclear.

Great advances have been made in *Wolbachia* genomics. The whole-genome sequence of *Wolbachia pipientis w*Mel strain from *D. melanogaster* provides an ideal system for studying the *Wolbachia–Drosophila* interactions [27]. The *w*Mel strain is a typical CI-inducing *Wolbachia* strain, belonging to Supergroup A based on gene sequencing and MLST typing [28]. It was successfully transferred into *Aedes aegypti* mosquitoes and blocked transmission of dengue [29, 30]. What's more, in an experimental transfection by microinjection, the *w*Mel strain established itself in a phylogenetically distant host insect *Bemisia tabaci* [31]. It is therefore intriguing to explore whether a *Wolbachia* strain derived from a phylogenetically distant host insect can also establish itself in *D. melanogaster*. Here we used a previously characterized *Wolbachia w*Ccep strain from the rice moth *Corcyra cephalonica* [31] to establish a *Drosophila*/*w*Ccep/*w*Mel system. Our purpose was to investigate the multiple interactions between the host and different *Wolbachia* strains and analyze the factors influencing the establishment of a *Wolbachia* strain in a novel insect host. We found that the *w*Ccep strain elicited a significant host immune response from the novel host, supporting the notion that the exogenous bacteria may trigger a robust innate immune response that eliminates the intruders [32]. Furthermore, based on *Wolbachia* titre measurement using RT-qPCR, the intrusion of *w*Ccep elicited an exclusion reaction from the native *w*Mel strain, inconsistent with the theoretical prediction that multiple infections favor cooperation between co-existing *Wolbachia* strains [33]. In the present study, we firstly transferred a *Wolbachia* strain derived from a distantly related host into *D. melanogaster*, which provides new insights into the multiple associations between the host and co-existing *Wolbachia* strains.

Materials and Methods

Insect Rearing and Wolbachia Isolation

The rice moth *C. cephalonica* was maintained on Maize-Rice bran–Sugar medium (25 °C, 65% RH and 14L:10D). The fruit fly *D. melanogaster* was maintained on Maize-Agarose-Yeast medium (25 °C, 60–70% RH and 14L:10D. The *w*Ccep strain was isolated from two moths using the Percoll density-gradient centrifugation method [31]. The purified bacteria were detected using the primers 81F/522R targeting *wsp* of Group B *Wolbachia* [34].

Microinjection

A volume of 46 nl bacterial suspension in SPG buffer (220 mM sucrose, 4 mM KH_2PO_4, 9 mM Na_2HPO_4, 5 mM L-glutamate, pH 7.4) was injected into the pupa of *D. melanogaster* using a glass needle on the platform of Nanoliter 2000 (World Precision Instruments, Sarasota, FL, USA). Approximately 100 pupae were injected, which were then placed in a climate incubator until eclosion (25 °C, 60–70% RH and 14L:10D). The newly emerged adults (G_0) were separately maintained in pairs (♀/♂) for establishing isofemale lines.

Quantitative Analysis of Wolbachia Titre

The relative titres of *w*Mel and *w*Ccep were measured using real-time quantitative polymerase chain reaction (qPCR) in *D. melanogaster* over 8 generations after microinjection. The primers 81F/522R specifically targeting *w*Ccep (B-*Wolbachia*) [34] and the primers *wsp*Q384/*wsp*Q513 targeting both *w*Mel and *w*Ccep [28] were used in qPCR analysis, with *GAPDH* as the internal reference (Table S1). The stability of primers was judged by the cycle threshold (C_t). Three adult flies were extracted for one DNA sample (50 ng/µl). The reaction was performed in a total volume of 20 µl containing 10 µl AceQ® qPCR SYBR® Green Master Mix (Vazyme, Nanjing, China), 0.4 µl of each primer (10 µM), 1 µl gDNA (50 ng) and 8.2 µl ddH_2O. The thermocycling program was 50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s. The relative titre was calculated using the $2^{-\Delta\Delta Ct}$ method [35]. All samples were assayed in triplicate on an ABI 7500 (Applied Biosystems, Carlsbad, CA, USA).

Crossing Experiments

The native strain was removed by tetracycline (0.25 mg/ml) for two consecutive generations. The uninfected flies were then injected with *w*Ccep solution (46 nl) and female isolines were constructed. Infected (IN) and uninfected (UN) flies from the 4th generation (G_4) were used for reciprocal crossing: UN♀ × UN♂; UN♀ × IN♂; IN♀ × UN♂, and IN♀ × IN♂. The newly emerged adults were used for mating in a tube (Φ2.2 cm) for 48 h, and the inseminated females were then placed individually in a petri dish (Φ3.5 cm). The number of eggs per female, hatching rate and developmental durations were calculated, and the level of CI was assessed according to the hatching rate of eggs.

Transcriptome Sequencing

The sequencing libraries were constructed from the pupae of fruit fly. Total RNA was extracted from approximately 30 pupae for each treatment: 24 h or 48 h post-injection (hpi) with *w*Ccep (46 nl) or the same volume of SPG buffer (negative control), with two repetitions. The cDNA libraries were established by Illumina Truseq RNA Sample Preparation Kit (NEB, San Diego, USA) with 2 µg RNA for each sample. Then, the Illumina MiSeq platform was used to produce 300-bp paired-end sequences. After the high-quality clean data were achieved, the genome sequences of *D. melanogaster* downloaded from NCBI (https://www.ncbi.nlm.nih.gov/genome/47) were used as the reference for identifying unigenes using Bowtie v2.0.6, TopHat v2.0.9 and HTSeq v0.5.4p3; the DESeq R package was used to characterize the differentially expressed genes (DEGs) (the corrected *P* value < 0.005; the $\log_2$ (fold change) > 1).

Functional Annotation of DEGs

The Gene Ontology (GO) enrichment analysis of DEGs [36] was conducted on the GO seq R package; the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis [37] was carried out using the KEGG orthology-based annotation system (KOBAS). The significantly enriched GO and KEGG pathway terms were determined using the hypergeometric test ($P < 0.05$). The DEGs related to host immunity, detoxification and stress responses against *Wolbachia* invasion were classified [4].

Association Analysis Between Gene Expression and Wolbachia Titre

Six treatments were carried out: 24 hpi with SPG buffer, 48 hpi with SPG buffer, 24 hpi with *w*Ccep, 48 hpi with *w*Ccep, 24 hpi with twice *w*Ccep (48 h apart), and 48 hpi with twice *w*Ccep (48 h apart). The expression of *attacin-D* and *edin* and the titre of *w*Ccep were measured using real-time qPCR, but the DNA templates were different: cDNA for the former and gDNA for the latter. Total RNA and gDNA were successively isolated from approximately 30 whole fruit flies using Trizol (TransGen Biotech, Beijing, China) [38]. The cDNA was synthesized using 0.5 µg total RNA and reverse transcriptase (HiScript® II One-Step RT-PCR Kit, Vazyme Biotech, Beijing, China) according to the supplier's instructions. The primers used (Table S1), the reaction system and thermocycling program for qPCR analysis were the same as described above. A linear regression analysis was performed to identify the association between gene expression and *Wolbachia* titre.

Data Analysis

The statistical differences were analyzed using One-way AVOVA followed by Student Newman Keuls (SNK) test at 0.05 and 0.01 levels on SPSS v.20.0 (SPSS Inc., Chicago,

IL, USA). Linear regression analysis was performed on Microsoft Excel v.1903.

Results

Quantitative Analysis of Wolbachia Titre

The results showed that the reference *GAPDH* was quite stable over different generations (Fig. S1). Quantitative analysis indicated that *w*Ccep could be transmitted over four generations but it was undetectable after G_4 (Fig. 1). Specifically, the titre of *w*Ccep climbed during the early stage after microinjection (24hpi-G_0), but then declined rapidly, even undetectable at G_3; surprisingly, it showed a sudden rebound at G_4, but then returned to an undetectable level (G_5 and later). In comparison, *w*Mel dropped immediately (24–48 hpi) and remained at a low level till G_4, but then began to rise (G_5 and later).

Crossing and CI

The crossing experiments showed that there was no significant difference in the developmental durations among different crossing types (SNK, $P=0.731$) (Table S2); no significant difference was observed in the number of eggs laid per female (SNK, $P=0.662$). However, a highly significant difference existed in the hatching rate between UN♀×IN♂ and the other crossing types (SNK, $P<0.001$) (Table 1). The significantly lower hatching rate in UN♀×IN♂ indicated a strong CI induced by *w*Ccep.

Host Responses to Wolbachia Intrusion

Transcriptome sequencing identified 240 DEGs (173 upregulated; 67 downregulated) at 24 hpi; 295 DEGs (183 upregulated; 112 downregulated) at 48 hpi, and 497 DEGs (254 upregulated; 243 downregulated) when comparing 24 hpi with 48 hpi (Fig. S2). KEGG analysis of DEGs identified a variety of induced biological pathways. Interestingly, more pathways were activated at 48 hpi than at 24 hpi (Fig. S3). Functional annotations revealed that *w*Ccep intrusion elicited typical immune reactions, including the Toll and JAK/STAT signaling pathways (Table S3), humoral and cellular immunity (Table S4). The majority of antimicrobial peptides (AMPs) were downregulated, whereas the lysozymes were upregulated. In addition, host detoxification and stress responses were also regulated (Table S5). The raw sequence data are available upon request.

Table 1 Fecundity and hatchability in different crossings between *w*Ccep-infected (IN) and antibiotic-treated uninfected (UN) fruit flies

Cross type (♀×♂)	No. of crosses (*n*)	No. of eggs per female	Percentage of hatchability (%)
UN×UN	11	92.33 ± 2.73^{a}	89.57 ± 1.04^{A}
UN×IN	9	92.67 ± 3.84^{a}	67.93 ± 1.65^{B}
IN×UN	10	87.75 ± 2.17^{a}	86.35 ± 0.49^{A}
IN×IN	10	91 ± 4^{a}	86.69 ± 2.78^{A}

The fruit flies are taken from G_4. The data are represented as means ± SE. The same lowercase and uppercase letters indicate no significant difference at $P<0.05$ and $P<0.01$ levels, respectively, and different uppercase letters indicate significant difference at $P<0.01$ level using one-way AVOVA followed by Student Newman–Keuls (SNK) test

Association Between Expression of Attacin-D/Edin and Wolbachia Titre

The results showed that the gene expression and *Wolbachia* titre varied considerably among different treatments (Table 2; Fig. S4). A general trend was that the

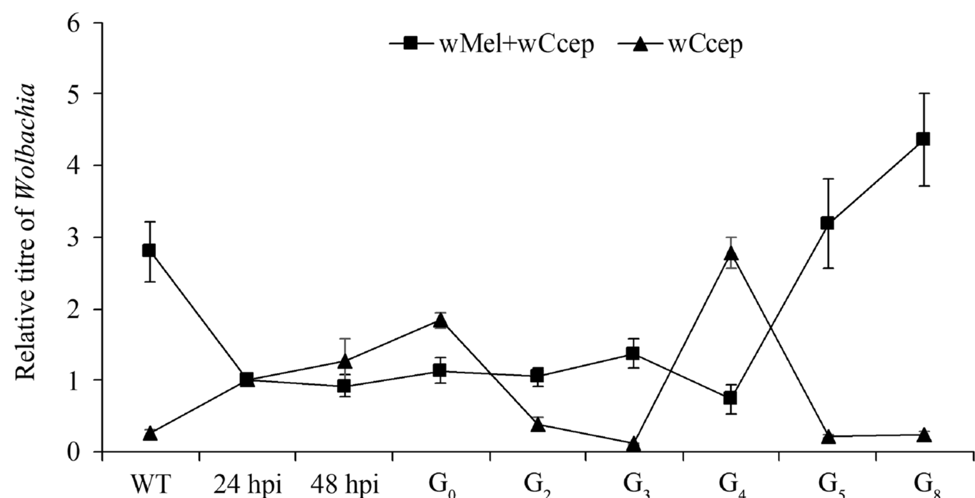

Fig. 1 The dynamics of the titres of *w*Mel and *w*Ccep strains in *D. melanogaster* during different stages after transinfection. Data are represented as means ± SE of three repetitions. *WT* wildtype, *hpi* hours post-injection

Table 2 The relative expression levels of *attacin*-D and *edin* and the relative titres of *w*Ccep strain in *D. melanogaster* under different treatments

	Relative expression of *attacin*-D	Relative expression of *edin*	Relative titre of *w*Ccep
24 hpi with SPG	1.505 ± 0.059^{Aa}	1.451 ± 0.247^{Aa}	0.368 ± 0.064^{De}
48 hpi with SPG	1.288 ± 0.058^{Aa}	1.635 ± 0.205^{Aa}	0.295 ± 0.056^{De}
24 hpi with *w*Ccep	1.057 ± 0.059^{Ab}	1.002 ± 0.032^{Ab}	1.081 ± 0.086^{Cd}
48 hpi with *w*Ccep	0.547 ± 0.077^{Bc}	0.839 ± 0.034^{Bc}	2.754 ± 0.332^{Bc}
24 hpi with twice *w*Ccep	0.569 ± 0.047^{Bc}	0.610 ± 0.022^{Bd}	6.307 ± 0.234^{Ab}
48 hpi with twice *w*Ccep	0.180 ± 0.112^{Cd}	0.087 ± 0.016^{Ce}	8.697 ± 0.700^{Aa}

Data are means ± SE of three repetitions. The different lowercase and uppercase letters within the same column indicate significant difference at $P < 0.05$ and $P < 0.01$ levels, respectively, using One-way ANOVA followed by Student Newman–Keuls (SNK) test. hpi, hours post-injection; 24 hpi and 48 hpi with twice *w*Ccep: twice injections are performed 48 h apart

injection of *w*Ccep downregulated gene expression; surprisingly, twice injection of *w*Ccep drastically reduced the expression of *attacin-D* and *edin*. Linear regression indicated that a significant negative correlation existed between the titre of *w*Ccep and the expression of *attacin*-D ($R^2 = 0.8157$; $P = 0.00082$) and *edin* ($R^2 = 0.8825$; $P = 0.00034$) (Fig. 2).

Discussion

Our studies suggested that the exogenous *w*Ccep strain from the moth could infect the fruit fly *D. melanogaster* and induce a high level of CI, but could only be transmitted for four generations (G_0 to G_4) in the novel host. Moreover, the intruding *w*Ccep strain should have suffered a competitive

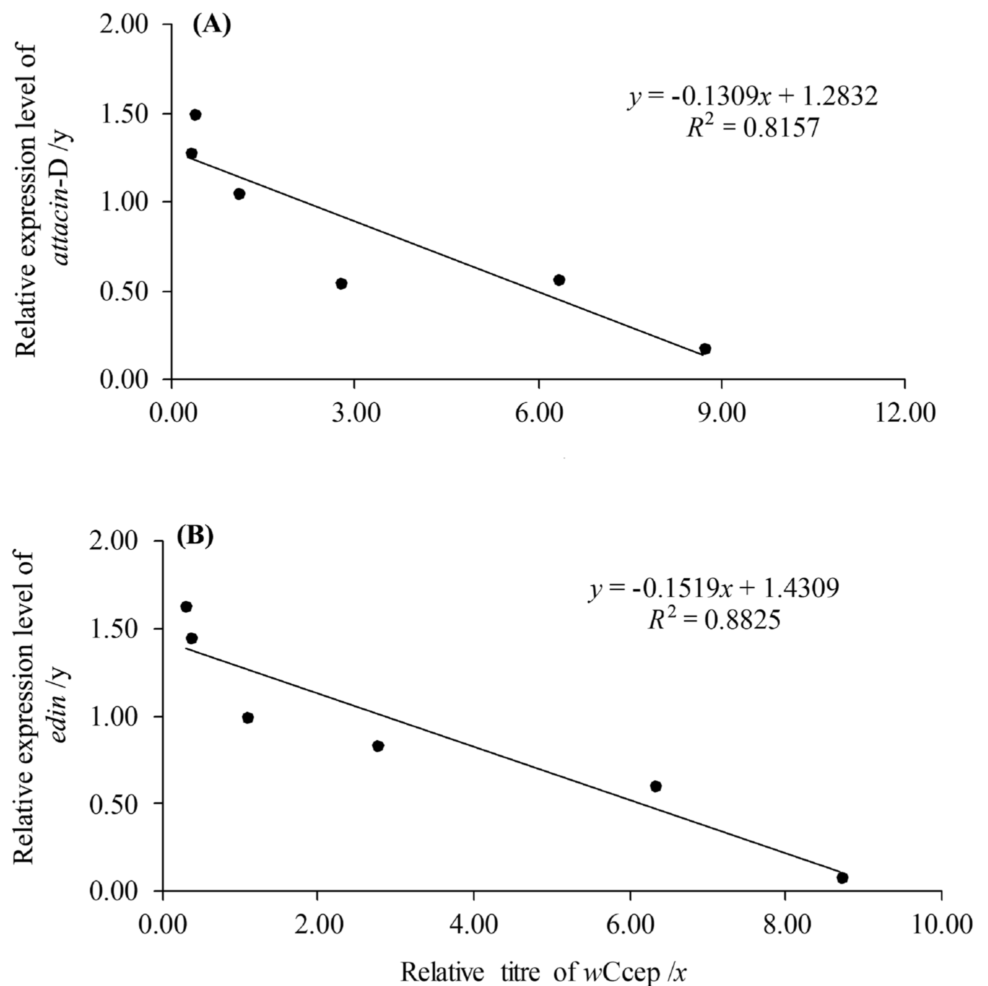

Fig. 2 Linear regression analysis between the relative titre of *w*Ccep strain and the relative expression level of *attacin*-D (**a**) and *edin* (**b**) in *D. melanogaster*

exclusion from the native *w*Mel strain, as their tires were negatively correlated during the invasion process. Furthermore, the recipient host imposed a remarkable immune suppression against the *w*Ccep strain. All of these reactions caused the failure of the establishment of *w*Ccep in *D. melanogaster*.

Although *Wolbachia* are common in *Drosophila*, the mechanisms underlying the host-*Wolbachia* interactions are only partially understood due to its unculturability. Microinjection is an ideal method for deciphering the interactions between the host and *Wolbachia* [39]. The *w*Ccep strain native to the rice moth *C. cephalonica* had previously been shown to establish itself in the hemipteran pest insect *B. tabaci* through microinjection [31]. *D. melanogaster* is known to harbor the *w*Mel strain [27], and thus it is expected that the invading *w*Ccep strain should actively interact with the novel host and native *w*Mel strain [40, 41]. The interactions may be viewed from the change in the titre of *Wolbachia* and the expression levels of immune genes. Our quantitative analysis of *Wolbachia* titre showed that *w*Ccep was negatively correlated with *w*Mel in their titres, indicating that there might exist a competitive relationship between the two co-existing strains. Indeed, the existence of a competition between the novel and native strains can partially explain why *w*Ccep could only be transmitted in the new host for a relatively short period of time (four generations). Several previous studies investigating the interactions between co-existing *Wolbachia* strains by comparing their titres (or densities) achieved mixed results: *Wolbachia* titre was highly strain-specific and unaffected by the presence of other strains in some parasitoid wasps and moths [42], whereas competition obviously existed between co-occurring strains in the beetle *Callosobruchus chinensis* and *Acromyrmex* leafcutter ants [43–45]. These results suggest that the interactions between the invading strain, host insect and native strain may be influenced by a complex of factors that need to be identified.

To investigate the effects of *w*Ccep on host developmental duration, fecundity (fitness effects) and CI level, crossing experiments were conducted using flies treated with antibiotics to obviate the effect of the native strain and then injected with or without *w*Ccep. The results suggested that *w*Ccep could induce a strong unidirectional CI in *D. melanogaster*, confirming the infection capability of *w*Ccep. Crossings also indicated that *w*Ccep infection imposed no significant fitness costs on the host as no obvious changes were observed in the developmental durations and the number of eggs laid per female. This is consistent with our previous results achieved in *B. tabaci*, where transinfection of *w*Ccep had no significant effect on the fecundity of the whitefly [31].

Transcriptome sequencing via RNA-seq coupled with functional annotations identified a host of genes involved in insect-*Wolbachia* interactions, including humoral and cellular immune responses, detoxification and stress resistance. One interesting finding is that sampling at 48 hpi identified more DEGs, while no substantial change was detected at 24 hpi. Another finding is that many immune-related DEGs (including the majority of AMPs such as *attacin*-D) were downregulated in response to *w*Ccep infection. One possible explanation is that the host has shut down these genes to provide protection for the native *Wolbachia* strain due to unknown fitness-related benefits. Nevertheless, massive doses of exogenous *Wolbachia* might be a possible factor causing the apparent suppression of many immune-related genes as observed in *Aedes albopictus*, *D. melanogaster*, *D. simulans* and *Tetranychus urticae* [46–48]. It seems that the immune- and stress-related genes played a subtle role in regulating the host insect-*Wolbachia* associations. This is further supported by our association analysis between the expression of *attacin*-D/*edin* and the titre of *w*Ccep, in which the expression of *attacin*-D/*edin* was significantly negatively correlated with the titre of *w*Ccep. This finding revealed that *attacin*-D/*edin* are two determinants of *w*Ccep titre. Considering Attacin-D and Edin are key components of insect innate immunity, our results suggest that Attacin-D and Edin play important roles in the host defense against the invading *Wolbachia* strain.

From an evolutionary perspective, coevolution is expected to favor low fitness cost, low level of CI, and high transmission rate. Conversely, the intrusion of an exogenous bacterial strain (e.g., injection of *Wolbachia*) into a novel host is expected to lead to negative fitness effect, high CI level, and low transmission rate [49]. In the present study, we did not measure the transmission rate, but the neutral fitness effect and high CI level measured for *w*Ccep are partially in agreement with the theoretical prediction. Thus, for future research, the *Drosophila*/*w*Mel/*w*Ccep system is expected to be useful for investigating the coevolution between *Drosophila* and *w*Mel, the competitive interaction between *w*Mel and *w*Ccep and the functional genes involved in the defense and anti-defense interactions between *Drosophila* and *w*Ccep.

In conclusion, the *w*Ccep strain can induce a high level of CI in the phylogenetically distant host *D. melanogaster* after infection, but can only be transmitted in the novel host for several generations. The invading *Wolbachia* strain imposed no significant fitness costs on the novel host, but suffered a robust immune response from the host and incurred a competitive exclusion from the native *Wolbachia* strain, which resulted in the failure of its establishment in *D. melanogaster*. Our data indicate that *D. melanogaster* and *w*Mel might have established a symbiotic relationship after a long-term coevolution.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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