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ARTICLE

Wolbachia supplement biotin and riboflavin to enhance reproduction in planthoppers

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Abstract

Symbiont-mediated nutritional mutualisms can contribute to the host fitness of insects, especially for those that feed exclusively on nutritionally unbalanced diets. Here, we elucidate the importance of B group vitamins in the association of endosymbiotic bacteria *Wolbachia* with two plant-sap feeding insects, the small brown planthopper, *Laodelphax striatellus* (Fallén), and the brown planthopper, *Nilaparvata lugens* (Stål). Infected planthoppers of both species laid more eggs than uninfected planthoppers, while the experimental transfer of *Wolbachia* into uninfected lines of one planthopper species rescued this fecundity deficit. The genomic analysis showed that *Wolbachia* strains from the two planthopper species encoded complete biosynthesis operons for biotin and riboflavin, while a metabolic analysis revealed that *Wolbachia*-infected planthoppers of both species had higher titers of biotin and riboflavin. Furthermore, experimental supplementation of food with a mixture of biotin and riboflavin recovered the fecundity deficit of *Wolbachia*-uninfected planthoppers. In addition, comparative genomic analysis suggested that the riboflavin synthesis genes are conserved among *Wolbachia* supergroups. Biotin operons are rare in *Wolbachia*, and those described share a recent ancestor that may have been horizontally transferred from *Cardinium* bacteria. Our research demonstrates a type of mutualism that involves a facultative interaction between *Wolbachia* and plant-sap feeding insects involving vitamin Bs.

Introduction

The intracellular bacteria *Wolbachia* infect a wide variety of invertebrates [1] and contain many supergroups [2–4], which are remarkably different in their biology and host distribution. Most insect *Wolbachia* strains, belonging to supergroup A or B [2], are facultative symbionts, with a few exceptions in bed bugs and parasitoid wasps [5, 6]. As parasites, *Wolbachia* have been known to manipulate host reproduction in order to facilitate their maternal transmission through inducing male-killing, feminization, parthenogenesis, and cytoplasmic incompatibility (CI) [7]. Recently, extensive evidence also shows that *Wolbachia* can benefit a number of insects as mutualists (reviewed in [8]). For example, *Wolbachia* can protect arthropod hosts against a variety of pathogens and abiotic stresses [9–11]. Some *Wolbachia* are also essential for successful egg development, such as in bed bugs, parasitic wasps and collembolan species [5, 6, 12, 13], and some *Wolbachia* can enhance the fecundity of female host insects [5, 14–16]. For instance, in the parasitoid wasp, *Asobara tabida*, *Wolbachia* strain *w*Atab3 (supergroup A) is required for oogenesis and is obligatory for female reproduction [5]. *Wolbachia*-infected (WI) females lay significantly more eggs than *Wolbachia*-uninfected (WU) individuals in *Aedes albopictus* mosquitoes, *Drosophila melanogaster*, and Psocoptera booklice [14–16]. However, the molecular mechanisms underlying *Wolbachia*-mediated fecundity enhancement are not fully elucidated.

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For insects that feed exclusively on certain types of diet (such as blood, plant-sap, and grain), insects cannot obtain complete nourishment due to some nutrients (like essential amino acids and most vitamins) being missing from food sources. As a result, those insects rely on symbiotically-mediated supplementation from microbial partners [17]. The vitamin Bs are water-soluble organic micronutrients that function as coenzymes in insects and other animals. The significance of vitamin B supplementation by symbionts has been well established, particularly in blood-feeding insects [18]. For instance, the tsetse fly, *Glossina morsitans*, relies on their obligatory symbiont, *Wigglesworthia*, to synthesize pyridoxamine (B6) and folate (B9) [19, 20]. The obligatory symbiont, *Francisella*, contributes to the fitness of African soft tick, *Ornithodoros moubata*, by synthesizing biotin (B7), riboflavin (B2), folate, and the cofactors coenzyme A and flavin adenine dinucleotide [21]. The obligatory symbiont, *Wolbachia* (supergroup F), synthesizes biotin and riboflavin to increase the fitness of the bed bug, *Cimex lectularius* [6]. In contrast to essential amino acids, vitamin Bs were thought to be unimportant nutrition factors for plant-sap feeders until recent evidence from metagenomics studies in aphids and experimental studies in red cotton bugs showed that vitamin Bs contributed to host survival and development [17, 22].

The small brown planthopper, *Laodelphax striatellus* (Fallén) (Delphacidae), and brown planthopper, *Nilaparvata lugens* (Stål) (Delphacidae), are two of the most destructive insect pests on rice that feed exclusively on plant-sap. Large populations of planthoppers cause a non-contagious plant disease, named "hopperburn", which causes the damaged leaves to become brown and dry, affecting plant yield [23]. It is estimated that, since the 1980s, the occurrence area of *N. lugens* covered about 50% of the area of cultivated rice, and caused 0.3 billion dollars economic loss per year [24]. Previous studies have showed that planthoppers harbor primary yeast-like symbiont (YLS) that may provide essential amino acids to host insects [25]. However, YLS genomes do not have complete vitamin B synthesis genes. In *N. lugens*, genomic analysis showed a symbiont, *Candidatus* Arsenophonus nilaparvatae (*Arsenophonus* hereafter) encodes genes needed for vitamin B biosynthesis [26]. Individuals infected with *Wolbachia* (53%) usually do not harbor *Arsenophonus* [27]; *L. striatellus* lacking *Arsenophonus* are more than 90% infected with CI-inducing *Wolbachia* [28–31].

Previously we reported that *Wolbachia* enhance host fecundity in planthoppers [29, 32]. In this study, we confirmed the positive effects of *Wolbachia* on female fecundity of *L. striatellus* and *N. lugens*, and demonstrated that *Wolbachia*-mediated provision of biotin and riboflavin is critical for both rice planthoppers by using a combination of experiments and genomic analyses.

Materials and methods

Insect rearing

The *L. striatellus* and *N. lugens* planthoppers were collected in rice farms at Nanjing and Sanya, China, respectively. Because of the almost 100% *Wolbachia* infection rate in *L. striatellus* [31], we first obtained WU planthoppers using tetracycline treatment (1 mg/ml) [30] and then microinjected *Wolbachia* from wild-type individuals back to WU planthopper embryos/nymphs (Fig. S1, Supplementary Materials and Methods). After confirming that the new-established *L. striatellus* line was reinfected with *Wolbachia* and behaved similarly to naturally-infected lines through their effects on host planthopper fecundity (Figs. S1 and S2), we used this reinfected line for all further comparisons to assess *Wolbachia* effects.

The infection rate of *Wolbachia* in *N. lugens* was around 50% [27]. WI and WU *N. lugens* were then isolated from natural populations and their nuclear genetic background was homogenized by backcrossing for at least seven generations. All planthoppers were maintained on rice seedlings in a climate-controlled room (25 °C, 60% RH, and a photoperiod of 8D: 16L [dark: light]). Before experiments, *Wolbachia* infection status in planthoppers was checked by PCR amplification of *Wolbachia* surface protein (*wsp*) gene with primers (81F/691R) [33]. Diagnostic PCR with specific primers showed the infection rate of *Aresnophonus* in *N. lugens* populations (either WI or WU insects) was 0%. Planthopper lines were further maintained for over 20 generations.

Fecundity assay

A group of 50 new nymphs were reared under different temperature conditions on rice seedlings. When the nymphs developed into adults, one virgin female (1-day-old) was placed in an oviposition chamber at room temperature (25 °C) to mate with two virgin males for 3 days, and the males were then removed after mating. Female fecundity was recorded daily in the next 2 weeks, with 30 replicates performed for each treatment. The temperature conditions for assays were 20, 25, and 27 °C for *L. striatellus* and 20, 25, and 30 °C for *N. lugens*.

Wolbachia density quantification

The abdomens or ovaries of planthopper nymphs were dissected in PBS. The genomic DNA was extracted with a Wizard Genomic DNA Purification Kit (Promega, Fitchburg, WI, USA). The relative *Wolbachia* density was measured as previously described [29]. In brief, gene copies of *Wolbachia* surface protein (*wsp*) were quantified with

qPCR with SYBR Premix Ex Taq (Takara, Kusatsu, Shiga, Japan) using ABI 7300 Real-Time PCR system and normalized relative to single-copy genes of the planthopper (beta-actin for *L. striatellus* and 40S ribosomal protein S11 for *N. lugens*) (Table S1). Three technical replicates were done for each gene per sample.

Vitamin titer analysis

Planthoppers developing into 3rd instar nymphs were collected for vitamin analysis. Sample preparation was based on the method described previously [34] and outlined in "Supplementary Materials and Methods". In brief, the sample was quantitatively analyzed using the LC (Agilent, Santa Clara, CA, USA)-MS (Thermo Scientific, Waltham, MA, USA) system. Biotin and riboflavin were measured for quantification under the electrospray ionization-positive mode using D-biotin (for biotin) and $^{13}C_4$,$^{15}N_2$-riboflavin (for riboflavin) as an internal standard. All vitamin analyses were undertaken in triplicate.

Wolbachia gene expression analysis

Expression of *Wolbachia* biotin and riboflavin synthesis genes was quantified with reverse transcription quantitative PCR (RT-qPCR). Total RNA was extracted from pools of 3rd instar nymphs per sample using Trizol (Thermo Scientific, Waltham, MA, USA). RNA was reverse-transcribed into cDNA using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech, Nanjing, Jiangsu, China) following the manufacturer's instructions. After that, cDNA amplification was quantitatively assessed with SYBR Green dye using ChamQTM SYBR® qPCR Master Mix (Vazyme Biotech, Nanjing, Jiangsu, China) in a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The qPCR cycling conditions involved an initial step of 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The specificity of the amplified products was determined by the presence of a single peak in the melting curve. Each cDNA sample was quantified in duplicate for each gene. The planthopper 60S ribosomal protein L14 (*rpl14*) gene was chosen as the reference control. The $2^{-\Delta\Delta CT}$ method was used to quantify relative gene expression [35]. The efficiency of primers was checked by qPCR by diluting cDNA as a gradient. All primer sequences used in this study are shown in Table S1.

Nutrition supplement experiment

After hatching, planthopper nymphs were supplemented with artificial diets containing different doses of biotin and riboflavin (Table S2) in a rearing chamber under different temperatures. The emerged adults were then transferred to oviposition chambers under room temperature to measure female fecundity [36]. One end of the oviposition chamber was covered with a normal artificial diet sachet and the other side was filled with oviposition medium (5% sucrose and 0.004 M salicylic acid, pH 6.5). The rearing chamber, oviposition chamber, and artificial diets were developed by Fu et al. [37].

Statistical analysis

The number of deposited eggs, *Wolbachia* densities, and vitamin titers were analyzed with a Shapiro–Wilk normality test followed by parametric or nonparametric analysis depending on whether data were normally distributed. For parametric analysis, Student's *t* test was used for the comparison of two treatments. One-way or three-way analysis of variances (ANOVA) with the Tukey post hoc tests were used for multiple treatment comparisons. For nonparametric analysis, we ran Wilcoxon rank-sum tests for comparisons of two treatments or Kruskal–Wallis tests with pairwise comparisons involving Bonferroni corrections where multiple treatments were compared. Multiple comparisons were completed using the "agricolae" package [38]. All statistical analyses were performed in R 3.5 [39].

Genome sequencing and vitamin gene annotation

Approximately 2000 adult planthoppers were collected for DNA extraction. The details of the extraction procedure can be found in the "Supplementary Materials and Methods". A combination of 454 pyrosequencing, Illumina sequencing, and targeted Sanger sequencing was performed to obtain the *w*Lug and *w*StriCN genomes in BGI Group (BGI, Shenzhen, Guangdong, China). The draft genome was generated by assembling Illumina reads into contigs and scaffolds with SOAPdenovo version 1.05 [40] and then by closing gaps with 454 reads and Sanger sequencing results of PCR amplicons. The assembled draft genomes were annotated using Prokka version 1.14 [41] with default parameters. Vitamin synthesis genes were annotated based on KO (KEGG Ortholog database) [42] and eggNOG database [43]. More details of gene prediction and annotation are provided in the "Supplementary Materials and Methods".

Molecular phylogenetic analysis

To assess the phylogenetic relationships of *w*Lug and *w*StriCN, we inferred their phylogeny using five multilocus sequence typing (MLST) genes (*gatB*, *coxA*, *hcpA*, *fbpA*, and *ftsZ*) and protein-coding sequences from all available genomes of *Wolbachia* A, B and F supergroups from NCBI (Table S6). The protein sequences were grouped with OrthoFinder version 2.2.6 [44], concatenated with FASconCAT-G version 1.04

[45], and trimmed with Gblocks version 0.91b [46] before alignment. Multiple sequence alignment was performed using MAFFT version 7.402 [47]. The maximum likelihood phylogenetic tree was constructed with IQ-TREE version 1.6.5 [48] using the best-fitting nucleotide substitution model (option "-m AUTO"). Node support was calculated with 1000 ultrafast bootstraps. The tree was visualized with FigTree software version 1.4.3 (https://github.com/rambaut/figtree/releases).

Evolution of biotin and riboflavin synthesis genes

The biotin and riboflavin synthesis pathway genes were identified with ortholog analysis by OrthoFinder version 2.2.6 [44] and a manual check of gene annotations from eggNOG database [43]. The biotin and riboflavin operon structures were visualized with the R package "genoPlotR" [49]. The distance matrix of concatenated biotin operon sequences was calculated with MEGA7 [50]. The comparison between the phylogenic trees was made with Dendroscope version 3.5.9 [51].

Results

Wolbachia stably enhance female fecundity in *L. striatellus* and *N. lugens*

We first compared the biological performance of the *L. striatellus* strain that was reinfected with *Wolbachia* after antibiotic curing with the naturally-infected *L. striatellus* strain and determined whether the new-established planthopper strain performed similarly to the naturally-infected planthoppers. We found that the newly-established WI planthoppers had a similar preoviposition period and deposited similar numbers of eggs compared with the natural WI planthoppers (100.1% of natural WI, $p = 0.997$, Fig. S1C). When compared with the tetracycline cured strain (WU), the artificially infected planthoppers (labeled as WI in Fig. 1a) had significantly higher fecundity at room temperature (146% of WU, $p < 0.001$, 25 °C, see also Fig. S1C). Therefore, *Wolbachia* appears to have a direct effect on fecundity.

Since *Wolbachia* density is sensitive to temperature [52, 53], we reared planthoppers under different temperatures, and then measured the correlation between female fecundity and *Wolbachia* density in hosts. The density of *Wolbachia* in *L. striatellus* was relatively smaller at lower temperatures (20 °C for nymph and 18 °C for adult) and higher at 25 °C (Figs. S3 and S4). The fecundity of WI planthoppers was also significantly reduced at lower temperature compared with that of planthoppers reared at room temperature (Fig. 1a), which indicates that fecundity changes are determined by *Wolbachia* density as well. Overall, regardless of the rearing temperatures, more eggs were deposited by WI females than WU females (Fig. 1a and Table S3). Similar to the pattern in *L. striatellus*, WI *N. lugens* laid more eggs than uninfected planthoppers at all temperatures (Fig. 1b). The proportion of eggs that hatched was similar between WI and WU planthoppers ($p = 0.9123$ for *L. striatellus* and $p = 0.8609$ for *N. lugens*, Fig. S5), suggesting the laid eggs were of similar quality. These results suggest that *Wolbachia* is beneficial for reproduction in both species independent of temperature conditions.

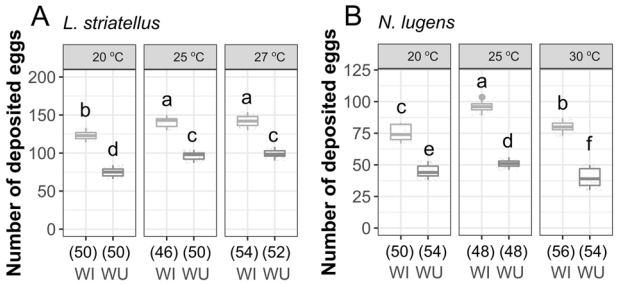

Fig. 1 Biological effect of *Wolbachia* on fecundity of *L. striatellus* and *N. lugens* at different temperatures. Number of deposited eggs for *L. striatellus* (**a**) and *N. lugens* (**b**). WI and WU represent infected and uninfected lines, respectively. The numbers of replicates are listed in parentheses under the treatments. Different letters indicate significant statistical differences at $p < 0.05$

Genome sequencing of *w*Lug and *w*StriCN

Given that the two *Wolbachia* have similar effects on their respective host species, we hypothesized that they might share similar genomic features which provide benefits for egg production. We combined Illumina, 454 and targeted Sanger sequencing data and obtained draft genomes of *w*Lug (*Wolbachia* from *N. lugens*) (Fig. S6A) and *w*StriCN (*Wolbachia* from *L. striatellus* China strain) (Fig. S6B and Table S4). The assembled genomes of *w*Lug and *w*StriCN contain two scaffolds and are 1.54 and 1.78 Mb, respectively (Table S4). The *w*Lug and *w*StriCN genomes have 1516 and 1747 coding DNA sequences, covering 84.51 and 85.34% of their genomes (Tables S5 and S6). Clusters of Orthologous Groups analyses showed similar gene function classifications between *w*Lug and *w*StriCN (Table S7). Phylogenetic analysis of genome-wide single-copy genes of insect-associated *Wolbachia* genomes and MLST genes allocated both *w*Lug and *w*StriCN to supergroup B (Figs. S7 and S8). Both analyses confirmed that *w*Lug and *w*StriCN cluster in different parts of the B group phylogeny.

Identification of vitamin B synthetic pathways from *w*Lug and *w*StriCN genomes

Previous studies showed that *Wolbachia* was essential for reproduction of the bloodsucking insect host via provisioning of B vitamins [34, 54]. Therefore, we focused on vitamin B biosynthetic pathways. By searching *Wolbachia*

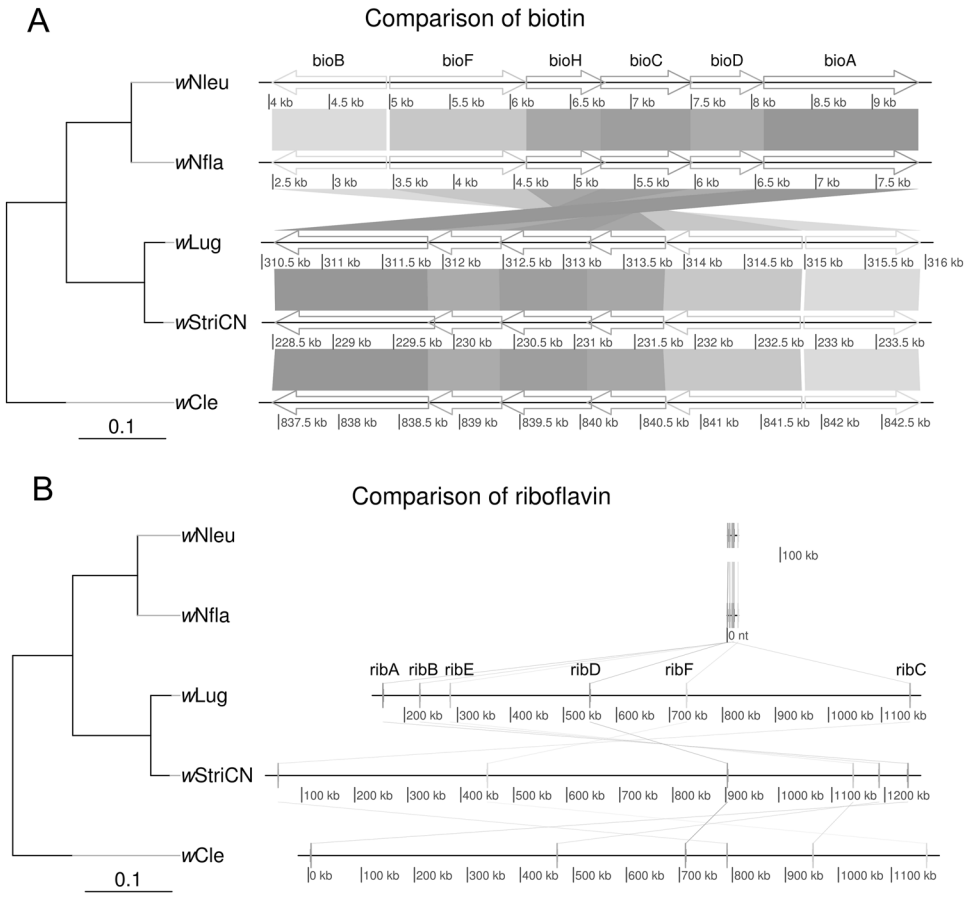

Fig. 2 Comparison of the organization of biotin (**a**) and riboflavin (**b**) synthesis genes in *Wolbachia*. The phylogeny of the only five *Wolbachia* strains that have complete biotin operons is shown. Bootstrap values of the Maximum Likelihood (ML) phylogenetic tree are 100% for all branches. Locations of vitamin B synthesis genes on the scaffolds are shown

encoded protein sequences against EggNOG and KEGG databases, we found the biosynthetic pathways for folate, pyridoxine, and thiamine in both *w*Lug and *w*StriCN genomes were incomplete (Table S8). Neither of the *w*Lug and *w*StriCN genomes had biosynthetic pathways for nicotinate and pantothenate (Table S8). However, we found complete biosynthetic pathways for biotin and riboflavin nutrition synthesis in both *w*Lug and *w*StriCN (Table S8). The biotin synthesis pathway includes genes *bioC, bioH, bioF, bioA, bioD*, and *bioB*. These genes locate closely together on the same position of the *w*Lug and *w*StriCN genomes (Fig. 2a). Based on the 30 analyzed *Wolbachia* genomes in this study, apart from *w*Lug and *w*StriCN, the complete biotin synthesis pathway has only so far been identified in *Wolbachia* of the bed bug *C. lectularius* (*w*Cle) [34], cuckoo bees *Nomada flava* (*w*Nfla) and *N. leucophthalma* (*w*Nleu) [55] (Table S9). Furthermore, we found *w*Lug and *w*StriCN encode compact operons of the riboflavin synthesis genes, which are *ribA, ribD, ribB, ribE, ribC*, and *ribF* (Table S9). Moreover, being different from *w*Nfla and *w*Nleu, riboflavin synthesis genes in *w*Lug and *w*StriCN are scattered on *Wolbachia* genome scaffolds (Fig. 2). From the genomic information obtained, we hypothesized that *Wolbachia* might provide biotin and riboflavin to *N. lugens* and *L. striatellus*.

The biotin and riboflavin titers are higher in WI *L. striatellus* and *N. lugens* individuals

To validate the hypothesis that *Wolbachia* provide most biotin and riboflavin in planthoppers, we quantified gene expression of *Wolbachia* biotin and riboflavin biosynthesis genes and measured biotin and riboflavin titers in WI and WU planthoppers. RT-qPCR analysis results showed the *Wolbachia* biotin and riboflavin biosynthesis genes were expressed much higher in WI than in WU planthoppers (Figs. S9 and S10). In addition, LC–MS results showed that the WI insects exhibited significantly higher titers of biotin and riboflavin in both species, indicating that both *w*Lug and *w*StriCN are capable of provisioning biotin and riboflavin (Fig. 3), which is concordant with the genomic data (Table S9). We note that biotin and riboflavin were not completely absent in WU planthoppers, suggesting that planthoppers may have access to other sources of vitamins.

Biotin and riboflavin rescue the fecundity deficiency in WU *L. striatellus* and *N. lugens*

Hypothesizing that riboflavin and biotin from *Wolbachia* may be important for fecundity in *L. striatellus* and

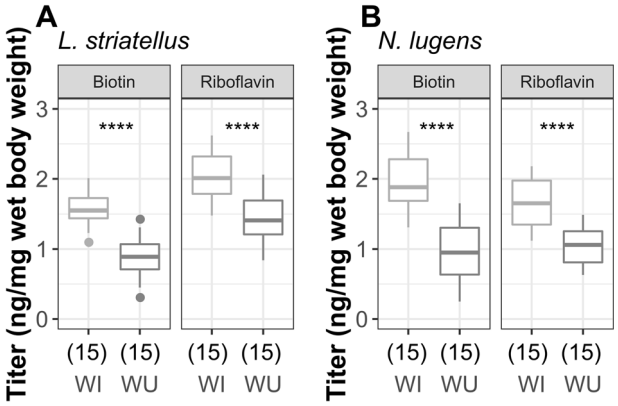

Fig. 3 Quantification of biotin and riboflavin in *Wolbachia*-infected (WI) and -uninfected (WU) *L. striatellus* and *N. lugens*. Vitamin quantities were compared with Wilcoxon rank-sum tests. The numbers of replicates are given in parentheses under the treatments. ****$p < 0.0001$

N. lugens, we undertook a three-factor experiment at three temperatures. We fed WI and WU planthopper nymphs on planthopper artificial diets containing different doses of biotin and riboflavin (Table S3) and measured the number of eggs laid after emergence. In concordance with results on rice plants, WI *L. striatellus*, and *N. lugens* exhibited higher fecundity than WU planthoppers on the normal artificial diet across all three temperatures tested for each species (Fig. 4). Three-way ANOVAs at each temperature showed that *Wolbachia* infection status, as well as biotin and riboflavin concentration, all significantly affected the fecundity of planthoppers. However there were significant interaction effects ($p < 0.001$, Table 1), reflecting the fact that combinations of the supplemented vitamins influenced the relative difference in fecundity between WI and WU.Note: * p < 0.05, ** p < 0.01, *** p < 0.001.

When biotin and riboflavin were excluded from the diet, we observed a significant decrease in the fecundity of *L. striatellus* and *N. lugens* (Fig. 4 and Table S3). Compared with WI planthoppers, WU planthoppers were particularly sensitive to the reduction of biotin; for both species, the lack of biotin resulted in no eggs being laid regardless of temperature (Fig. 4 and Table S3). The absence of riboflavin also led to a reduction in the fecundity of WU planthoppers across all treatments. In addition, the reduction in fecundity in the absence of biotin was minor in WI planthoppers (87% of normal for *L. striatellus* and 62% of normal for *N. lugens* at 25 °C) compared with the complete loss of fecundity (zero deposited eggs) for the WU planthoppers (Fig. 4 and Table S3).

We also analyzed whether supplementing vitamins can rescue the fecundity defect of WU planthoppers. For both *L. striatellus* and *N. lugens*, when WU females were reared on diets with more biotin, they laid significantly more eggs (156% of normal for WU *L. striatellus* and 151% of normal for WU *N. lugens* at 25 °C, Fig. 5 and Table S3). When reared on diets supplemented with both biotin and riboflavin, the fecundity of WU planthoppers was mostly recovered to that of WI planthoppers (97.6% of normal WI *L. striatellus* and 95.5% of normal WI *N. lugens* at 25 °C, Fig. 5b). For WI *L. striatellus*, the benefit of supplementation by biotin was significant under all three temperature conditions, but not always for riboflavin (Fig. 5a–c). In WI *N. lugens*, supplementation by biotin consistently increased fecundity (125% of normal at 25 °C), but supplementation by riboflavin decreased fecundity (49.0% of normal at 25 °C, Fig. 5d–f and Table S3). Taken together, the above results suggest that *Wolbachia*-provided biotin (and perhaps riboflavin as well) are important for female fecundity in *L. striatellus* and *N. lugens*.

Evolutionary origin of biotin and riboflavin operons in *w*Lug and *w*StriCN

Apart from *w*Lug and *w*StriCN, complete biotin synthesis pathways exist only in *w*Cle [34], *w*Nfla, and *w*Nleu [55] (Table S8), while riboflavin synthesis genes exist in most other insect-associated *Wolbachia* [54]. To understand the evolution of biotin and riboflavin synthesis genes in *Wolbachia*, we analyzed available complete and draft genomes of insect-associated *Wolbachia*. Phylogenetic analysis showed that both *w*Lug and *w*StriCN belong to supergroup B, while *w*Nfla and *w*Nleu belong to supergroup A and *w*Cle belongs to supergroup F (Fig. S7). The existence of similar biotin synthesis genes in distinct *Wolbachia* supergroups may reflect either horizontal transfer of biotin synthesis genes into the respective supergroups, or a large-scale gene loss in various distinct *Wolbachia* supergroups of an ancient biotin gene complex [55].

When we investigated the phylogenetic history of biotin synthesis genes across diverse bacteria, the incongruence between phylogenies of biotin operons and bacterial taxonomy supports the frequent horizontal transfer of biotin synthesis genes (Fig. 6). The distinct monophyletic clade of *Wolbachia* biotin genes implies a relatively recent acquisition. To identify putative donors of biotin operon genes, protein sequences of *Wolbachia* biotin synthesis genes were blasted against the NCBI "refseq_protein" database. The top blast hits were from the intracellular symbiont *Cardinium* (Table S10), which is consistent with the phylogenetic tree of biotin operon genes (Fig. 6).

In contrast, the riboflavin synthesis genes were conserved across most sequenced *Wolbachia* genomes [54] (Table S9). The phylogeny of riboflavin operons mirrored the *Wolbachia* genomic phylogeny to some extent (Fig. 7), suggesting that the riboflavin synthesis genes were present in a common ancestor of *Wolbachia*. Meanwhile, the incongruent phylogenies found within supergroups implies

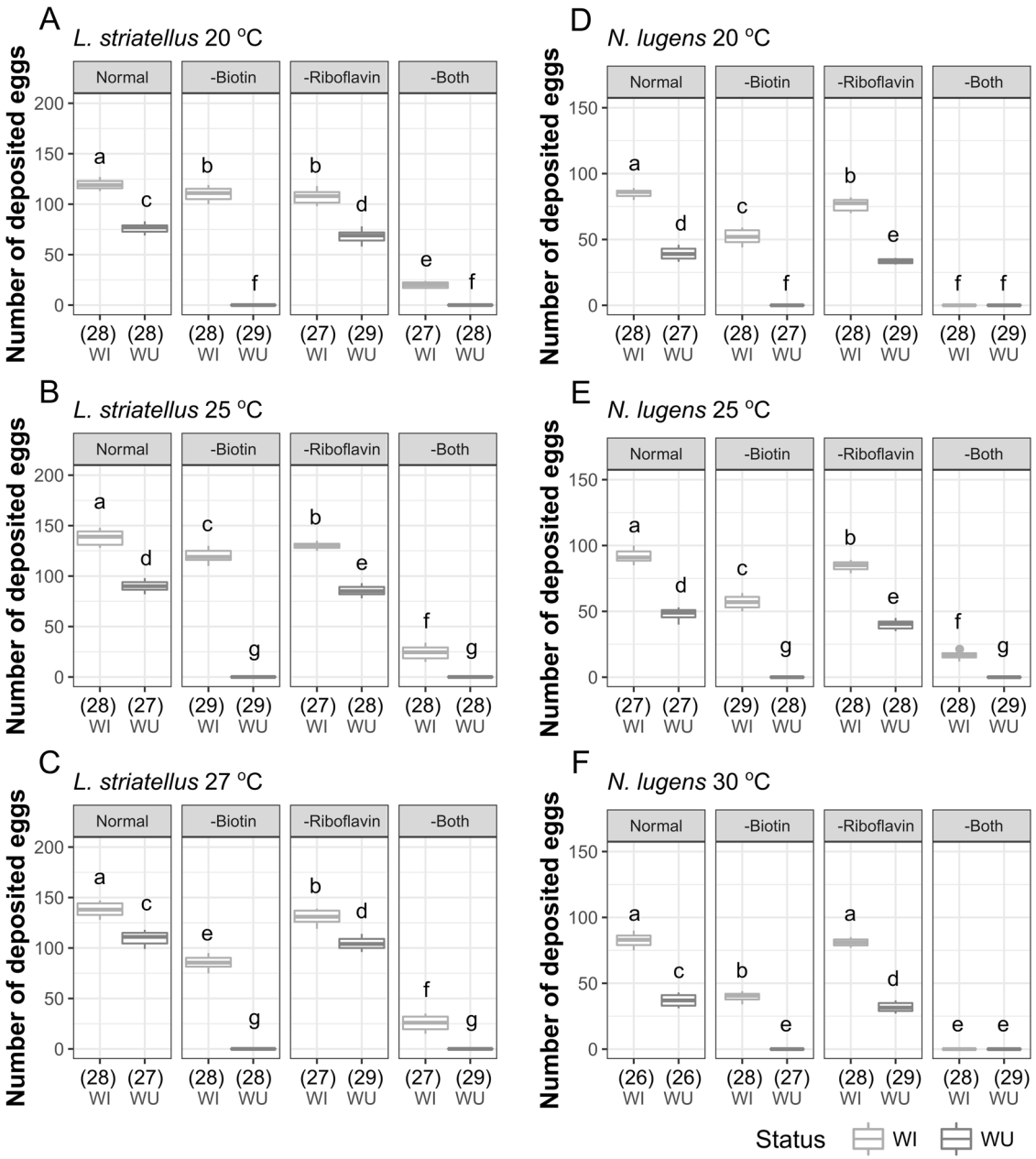

Fig. 4 Number of deposited eggs for *L. striatellus* and *N. lugens* reared on artificial diets supplemented with less biotin, riboflavin, and less biotin and riboflavin under different temperatures. **a**–**c** *L. striatellus* at 20, 25, and 27 °C; **d**–**f** *N. lugens* at 20, 25, and 30 °C. WI and WU represent infected and uninfected lines, respectively. Normal is the artificial diet that has a normal concentration (1×) of biotin and riboflavin. The other treatments (labeled with "−") lack the vitamins. The detailed recipes are shown in Table S2. The numbers of replicates are given in parentheses under the treatments. Different letters indicate significant statistical differences at $p < 0.05$

that vertical transmission is not strictly followed among closely-related *Wolbachia* strains [54].

Discussion

In this study, we investigated the mechanism of *Wolbachia*-mediated benefit in plant-sap feeding planthoppers. Our experimental and genomic evidence demonstrated that B supergroup *Wolbachia* enhance planthopper fitness, likely by synthesizing essential nutrients (biotin and riboflavin). We confirmed that both *w*Lug and *w*StriCN increase female fecundity in the Hemipteran planthoppers *N. lugens* and *L. striatellus* [29, 32], which was evident across a range of temperatures at which planthoppers were reared. Our data also showed that *Wolbachia* density in planthopper nymphs

Table 1 Three-way ANOVAs on factors influencing planthopper fecundity at each tested temperature

L. striatellus	20 °C			25 °C			27 °C		
Effects	df	Mean Sq	*F* value	df	Mean Sq	*F* value	df	Mean Sq	*F* value
Status	1	68152	2931.92***	1	46278	1739.10***	1	34189	1109.683***
Biotin	1	36526	1571.39***	1	44741	1681.35***	1	19904	646.02***
Riboflavin	1	278	11.97***	1	25	0.92	1	1904	61.81***
Status:Biotin	1	7960	342.44***	1	10788	405.39***	1	1611	52.27***
Status:Riboflavin	1	107	4.59*	1	169	6.37*	1	168	5.44*
Biotin:Riboflavin	1	21	0.92	1	1328	49.91***	1	223	7.25**
Status:Biotin:Riboflavin	1	277	11.93***	1	714	26.85***	1	11	0.36
Residuals	215	23		218	27		214	31	
N. lugens	20 °C			25 °C			30 °C		
Effects	df	Mean Sq	*F* value	df	Mean Sq	*F* value	df	Mean Sq	*F* value
Status	1	67554	3825.34***	1	50496	629.84***	1	47235	3127.04***
Biotin	1	79693	4512.76***	1	98210	1224.97***	1	61265	4055.86***
Riboflavin	1	70	3.99*	1	1043	13.01***	1	1192	78.94***
Status:Biotin	1	11158	631.82***	1	6398	79.80***	1	6393	423.22***
Status:Riboflavin	1	8606	487.34***	1	9378	116.97***	1	9023	597.33***
Biotin:Riboflavin	1	20449	1157.97***	1	18130	226.14***	1	13767	911.40***
Status:Biotin:Riboflavin	1	7478	423.44***	1	6880	85.82***	1	9643	638.42***
Residuals	214	18		214	80		209	15	

Note: *p < 0.05, **p < 0.01, ***p < 0.001.

was reduced under nonoptimal temperature conditions, with likely flow-on effects on the extent to which *Wolbachia* enhanced host reproduction.

Our genomic analysis, metabolic analysis and nutrient supplementing experiments indicate that the *Wolbachia*-mediated benefit in plant-sap feeding planthoppers involves provision of biotin and riboflavin. Biotin and riboflavin are vitamin B family members that function as coenzymes and are generally not synthesized by insects. Many insects, especially those who feed exclusively on a limited type of diet, derive their vitamin B requirements from their microbial symbionts [18]. For instance, the blood-feeding tsetse fly *G. morsitans* and African soft tick *O. moubata* rely on the symbionts *Wigglesworthia* and *Francisella* to synthesize vitamin Bs [19–21]. However, the importance of vitamin Bs for plant-sap feeders may be underestimated. Recently, a metagenomics study revealed that the primary symbiont of aphids, *Buchnera*, has lost the ability to synthesize riboflavin, while other coresident symbionts in aphids have intact routes for the biosynthesis of this compound [22]. Experimental studies showed that gut symbionts of red cotton bugs, *Dysdercus fasciatus*, contribute toward host fitness through the supplementation of vitamin Bs [17]. In combination with our results, it is becoming clear that vitamin Bs are important in plant-sap feeders.

Comparative genomic analysis of diverse insect-associated *Wolbachia* strains showed the synthesis pathway for riboflavin is highly conserved [54]. In contrast, the biotin synthesis pathway so far has only been detected in five *Wolbachia* strains, i.e., *w*Cle, *w*Nleu, *w*Nfla, *w*Lug, and *w*StriCN (Table S6). *w*Nleu and *w*Nfla were reported to encode complete biotin synthesis operons via genomic analysis [55], but no biological experiments have yet been completed. The biological roles of *Wolbachia*-provided riboflavin and biotin have been experimentally demonstrated for the *w*Cle infection in *C. lectularius*, a blood-feeding bed bug [6, 34, 54]. However, *w*Cle belongs to supergroup F, phylogenetically distant from most other insect-associated *Wolbachia* (Fig. S7). Moreover, *w*Cle is an obligate bacteriocyte-associated nutritional mutualist for the bed bug [6], whose localization, infection dynamics, and biological roles are more comparable to those of obligate mutualistic bacteriocyte symbionts like *Wigglesworthia* in tsetse flies and *Buchnera* in aphids. In our case, both *w*Lug and *w*StriCN are facultative symbionts and belong to supergroup B (Fig. S7). Given that supergroup B *Wolbachia* are prevalent in many arthropods living on limited types of diets [1], our findings raise the possibility of *Wolbachia*-provided nutritional mutualism in other insects.

Comparative genomic analysis indicate that biotin operons are found in five *Wolbachia* genomes scattered across

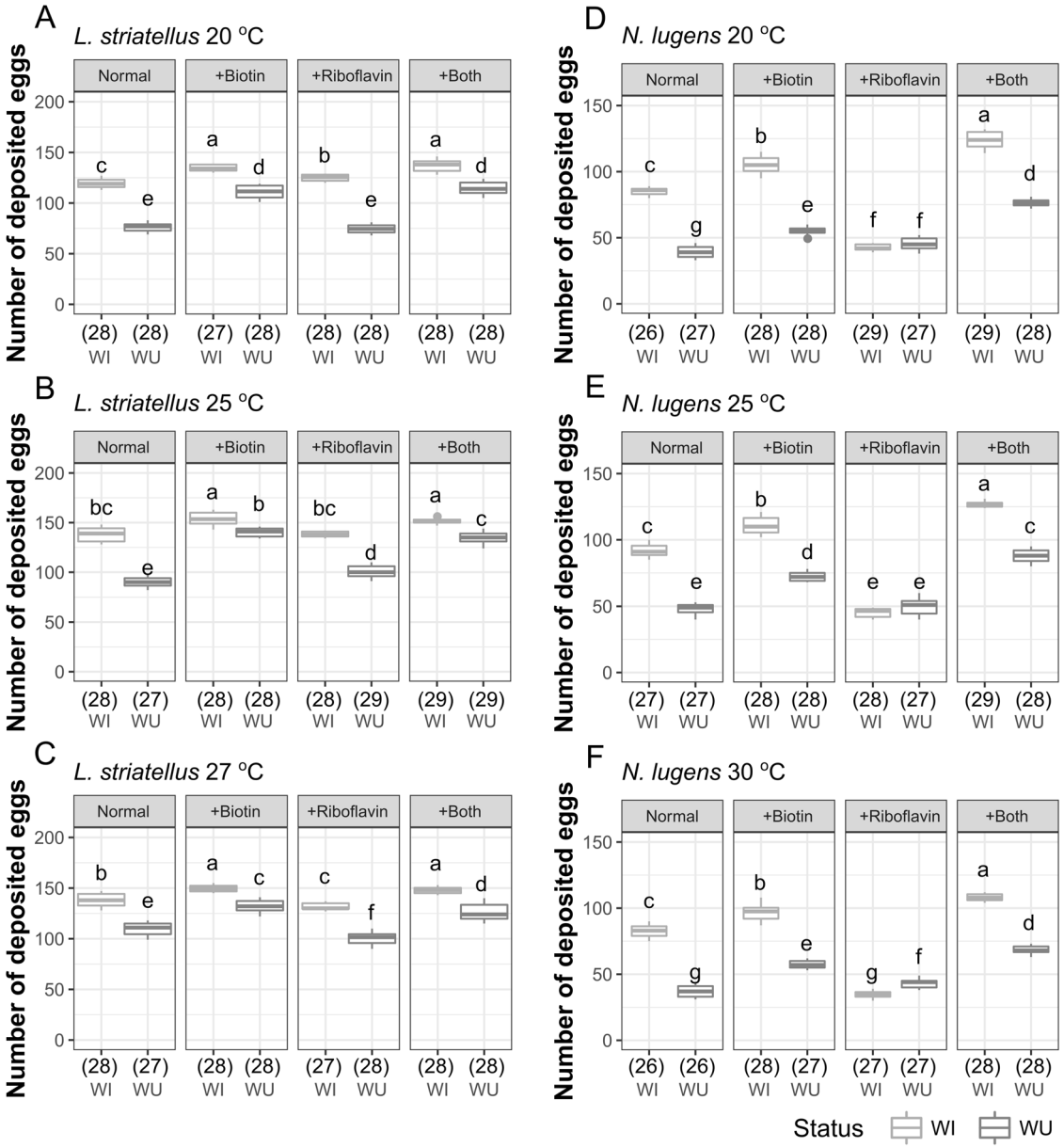

Fig. 5 Number of deposited eggs for *L. striatellus* and *N. lugens* reared on artificial diets supplemented with more biotin, riboflavin and more biotin and riboflavin under different temperatures. **a**–**c** *L. striatellus* at 20, 25, and 27 °C; **d**–**f** *N. lugens* at 20, 25, and 30 °C. WI and WU represent infected and uninfected lines respectively. Normal is the artificial diet that has a normal concentration (1×) of biotin and riboflavin. The other treatments (labeled with "+") contain 3× fold of the normal concentration of corresponding vitamins. The detailed recipes are shown in Table S2. The numbers of replicates are listed in parentheses under the treatments. Different letters indicate significant statistical differences at $p < 0.05$

three supergroups, supporting the notion that the biotin operons were possibly laterally transferred from another organism. The *Wolbachia* biotin synthesis genes formed a well-supported monophyletic group for the biotin operon phylogeny, suggesting the biotin operon might originate from the same ancestor [55]. BLAST results show that the most closely-related protein sequences of the biotin operon are from *Cardinium*, an intracellular endosymbiont [56]. *Cardinium* was reported to coinfect with *Wolbachia* in some planthoppers [31]. A recent genome analysis revealed that *Cardinium* from *Sogatella furcifera*, another rice planthopper, also harbors complete synthesis genes of biotin [57]. Therefore, we speculate that horizontal transfers of biotin synthesis genes might have occurred several times (in species coinfected by *Cardinium* and *Wolbachia*).

In contrast, riboflavin synthesis genes are likely to have been present in the ancestor of *Wolbachia*, as they are conserved in most *Wolbachia* genomes and evolved mostly convergent with *Wolbachia* phylogeny [54]. Riboflavin provisioning appears to be critical for *Wolbachia* and therefore

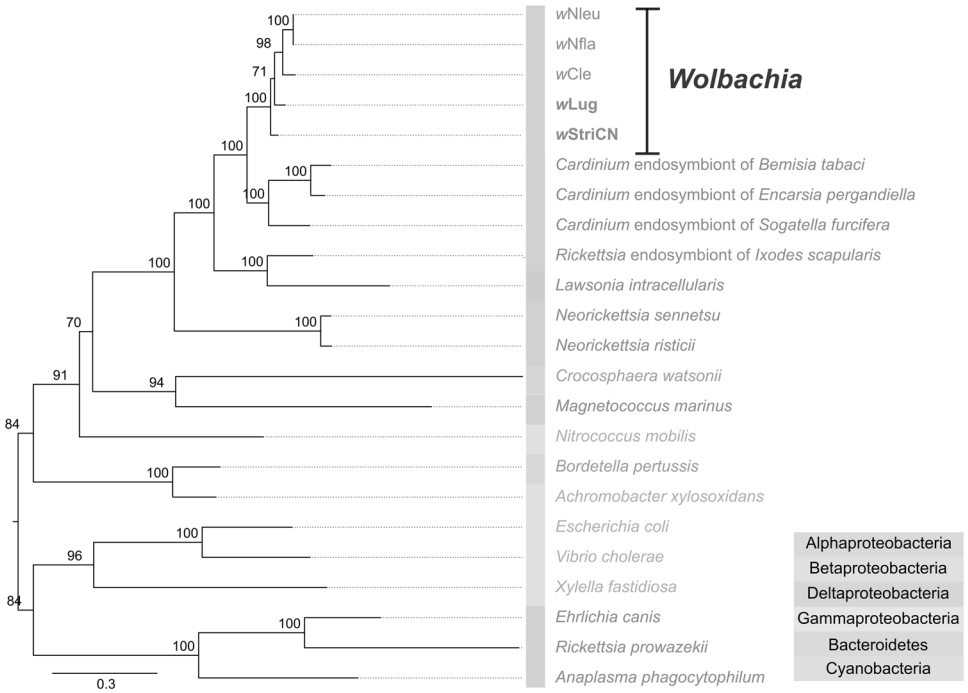

Fig. 6 Phylogenetic analysis of biotin synthesis genes of *Wolbachia*. The ML tree was calculated with a concatenated protein sequence (BioA, BioD, BioC, BioH, BioF, and BioB) of bacteria from six different phyla using an LG + I + G4 substitution model. Taxonomic affiliations are coded by color. The *Wolbachia* sequence obtained in this study is shown in bold. Bootstrap values are indicated at nodes (only values > 50% are shown). The GenBank accession numbers of proteins used in the analysis are listed in Table S9

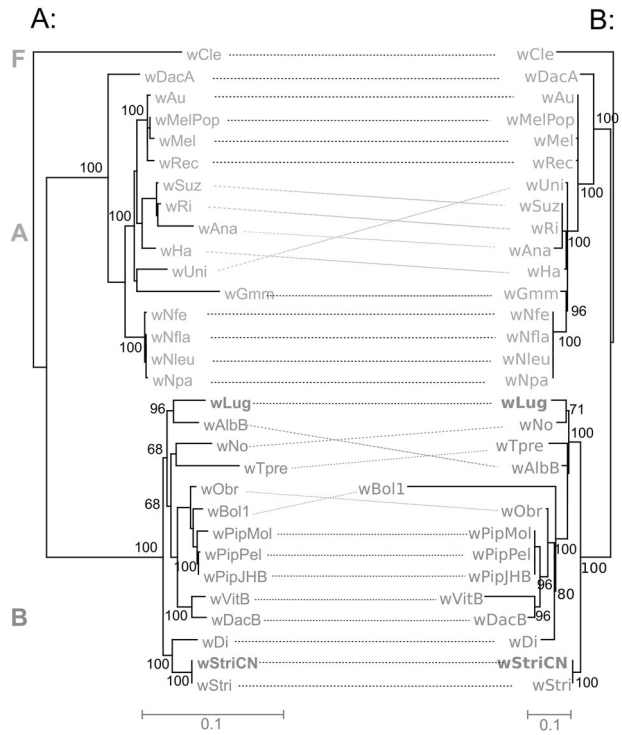

Fig. 7 Phylogenetic comparisons between *Wolbachia* core genes and riboflavin synthesis genes. **a** Phylogeny of *Wolbachia* supergroups A and B. The ML tree is based on concatenated protein sequences of 268 single-copy genes. **b** Phylogeny of riboflavin synthesis genes. The ML tree is based on concatenated protein sequences of ribA, ribB, ribD, ribC, ribE, and ribF. The trees were rerooted with the outgroup *w*Cle. Numbers on nodes correspond to bootstrap support values (>50%). The scale bar represents the average number of substitutions per site. Full names and accession numbers of *Wolbachia* strains are given in Table S9

maintained throughout diverse *Wolbachia* strains. Recent studies show *Wolbachia* infection levels decreased in mosquito cells lacking riboflavin [58], suggesting the importance of riboflavin for *Wolbachia*. Our data also showed that adding riboflavin increased the fecundity of WU planthoppers. However, though the mechanism is still unknown, this treatment resulted in adverse effects on WI *N. lugens*, suggesting a particular titer of this vitamin is required in *N. lugens*. Biotin supplementation was more critical for recovering host fitness in WU planthoppers than riboflavin supplementation (Fig. 4) and this also appears to be the situation in bed bugs [34, 54], pointing to the value of acquiring biotin synthesis operons horizontally for *Wolbachia* in these insects.

Rice planthoppers harbor primary mutualistic YLS. YLS synthesize all essential amino acids and ergosterol for planthoppers and are important for host fitness [25, 59, 60]. Previous studies have reported that high temperature can dramatically suppress the population of YLS and result in negative fitness consequences for host planthoppers [61]. In the current study, regardless of rearing temperatures, WI planthoppers consistently laid more eggs than the WU individuals, suggesting that the *Wolbachia*-mediated fecundity advantage is independent of temperature (and perhaps any effects of YLS). It is worth noting that genome analysis showed both YLS and *N. lugens* genomes had gene deficiencies in several vitamin B biosynthesis pathways [25]. Based on our results, we speculate that *Wolbachia*-provided vitamin Bs compensate with YLS to benefit rice planthopper fitness. On the other hand, field investigations showed that *N. lugens* individuals that lack *Wolbachia*

usually harbor *Arsenophonus* [27, 29]. Although no experiments have yet been undertaken, genomic analysis showed *Arsenophonus* encodes all genes needed for vitamin B biosynthesis [26], which may have similar nutritional mutualistic roles as *Wolbachia*.

Overall, our results demonstrate that two members of supergroup B *Wolbachia* can increase the fecundity of plant-sap sucking planthoppers. In addition, our results point to the beneficial effect of *Wolbachia*-synthesized biotin and riboflavin for plant-sap feeders. Removal of these bacteria from planthoppers might provide a new target for pest control. This is the first report of supergroup B *Wolbachia* acting as nutritional mutualists in plant-sap sucking insects, highlighting the importance of beneficial phenotypes across the *Wolbachia* phylogeny.

Data availability

The draft genome sequences data are freely available in NCBI GenBank under accession numbers MUIY00000000 (*w*Lug) and MUIX00000000 (*w*StriCN).

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Author contributions JFJ, XLB, and XYH designed the research; JFJ, XLB, YG, DSZ, KJZ, HJH, JTG, XZ, ZX, AH, and XYH performed the research; JFJ, XLB, DSZ, JJH, ZX, AH, and XYH wrote and edited the paper. All authors read and approved the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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