

# *Wolbachia* enhances ovarian development in the rice planthopper *Laodelphax striatellus* through elevated energy production

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The endosymbiont *Wolbachia* can both benefit host nutrition and manipulate host reproduction to its own advantage. However, the mechanisms of its nutritional benefits remain unclear. We show that *Wolbachia* enhances ovarian development in the small brown planthopper *Laodelphax striatellus* by boosting energy production. *Wolbachia*-infected females have increased fecundity, accelerated ovarian development, and prolonged oviposition. Enhanced activity of mitochondrial complex I is linked to increased ATP production and the expression of energy metabolism-related genes. We further identify that *Wolbachia*-synthesized riboflavin is crucial for ATP production and ovarian development. A riboflavin transporter, *slc52a3a*, positively correlates with *Wolbachia* density and is required for normal ovarian maturation. Our findings demonstrate that *Wolbachia*-produced riboflavin drives energy production and accelerates ovarian maturation, thus improving host fecundity. This research reveals insights into symbiont-host metabolic interactions and underscores the role of nutrient delivery in symbiosis.

The symbiotic relationship between insects and bacteria often exhibits a dual nature, wherein symbiotic bacteria can simultaneously benefit and harm their hosts, leading to their characterization as “Jekyll and Hyde” symbionts<sup>1–4</sup>. This duality highlights the complex and dynamic interactions between symbionts and their hosts. *Wolbachia* is one of the most notable examples. Although *Wolbachia* is known for its harmful reproductive manipulations, such as inducing male killing and cytoplasmic incompatibility (CI) that leads to offspring mortality<sup>5,6</sup>, recent studies have also revealed beneficial effects of *Wolbachia* on host fecundity in some insects<sup>7,8</sup>. For instance, *Wolbachia* has been shown to increase host fecundity under conditions of stress or nutrient deprivation and can be essential for oogenesis<sup>9–12</sup>. Despite these insights, the molecular mechanisms by which *Wolbachia* enhances host fecundity are not entirely clear.

Among the various mechanisms that promote fecundity, the role of *Wolbachia* in providing vitamin B to their hosts has attracted increasing interest. In the bed bug *Cimex lectularius*, *Wolbachia*

synthesize biotin (vitamin B7) and riboflavin (vitamin B2), which are essential for host growth and fecundity<sup>13,14</sup>. Comparative genomic studies show that the riboflavin synthesis pathway is highly conserved among different *Wolbachia* strains, underscoring its importance in host-symbiont interactions<sup>15</sup>. Riboflavin is a water-soluble molecule that plays a critical role in energy metabolism and cellular function. It is a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are essential cofactors for mitochondrial electron transport chain enzymes, and thereby supports energy production and oxidative phosphorylation<sup>16,17</sup>. However, little is known about the effects of riboflavin synthesized by *Wolbachia* on host energy function and reproduction.

*Wolbachia* induces complete CI while simultaneously enhances female fecundity in the small brown planthopper (*Laodelphax striatellus*, SBPH), a major rice pest that feeds exclusively on plant sap<sup>18,19</sup>. This dual functionality allows *Wolbachia* to establish and rapidly spread within SBPH populations, as evidenced by its near 100%

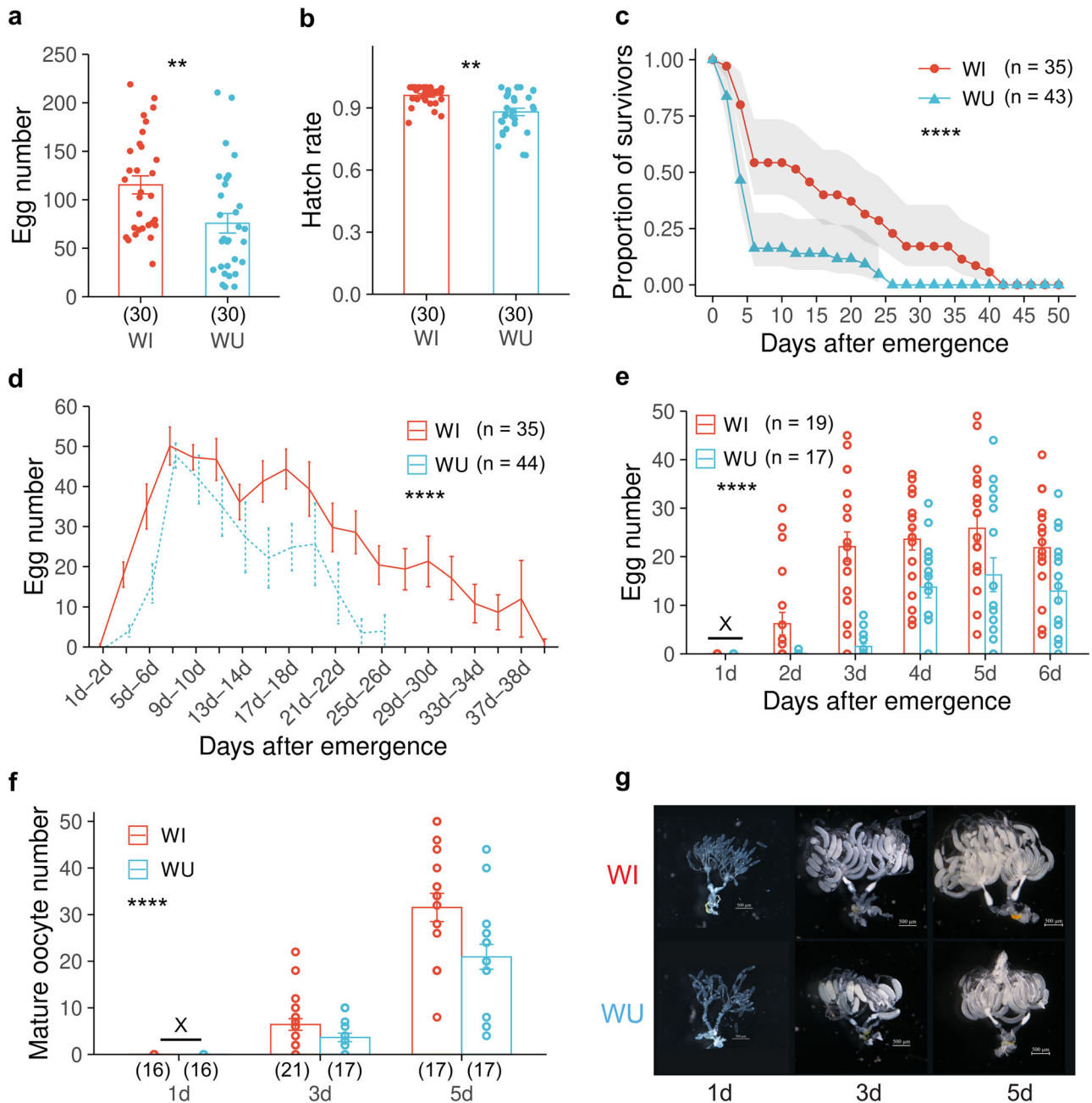
infection rate in field surveys<sup>20,21</sup>. Our previous work has shown that *Wolbachia*-provided biotin and riboflavin play significant role in promoting SBPH fecundity<sup>14</sup>. In this study, we combine transcriptomic analyses and experimental approaches to demonstrate the mechanisms by which riboflavin enhances reproductive capacity. We find that *Wolbachia* infection increases energy production in early adult SBPH, accelerating ovarian development and enhancing female fecundity. We further reveal that this effect is mediated by *Wolbachia*-synthesized riboflavin and the host's riboflavin transporter, *slc52a3a*. These

findings provide a clear understanding of the molecular and cellular mechanisms underlying *Wolbachia*'s reproductive contributions.

## Results

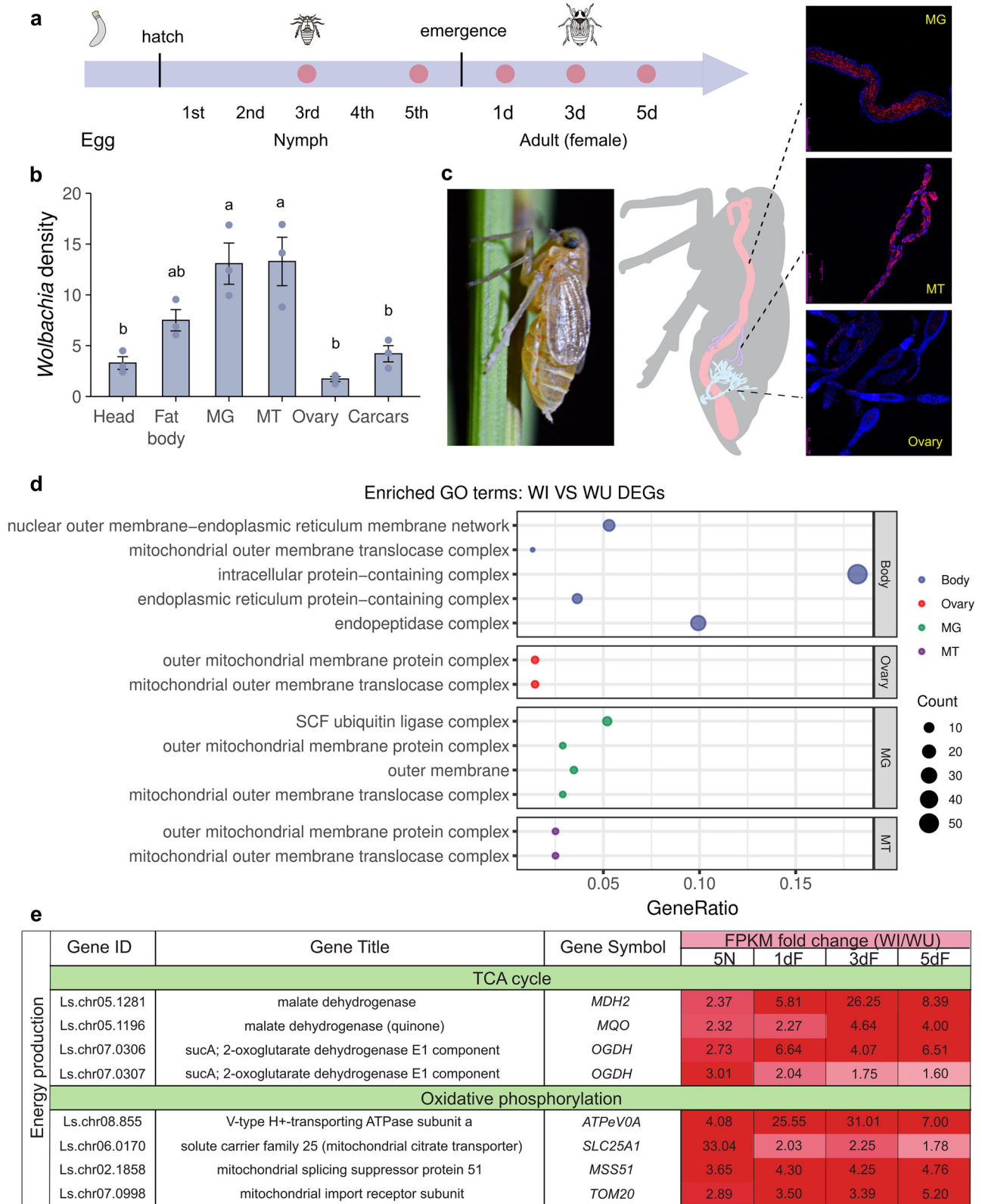
### *Wolbachia* enhances female ovarian development in SBPH

We first compared the biological performance of *Wolbachia*-infected (WI) and *Wolbachia*-uninfected (WU) SBPH (Fig. 1). *Wolbachia* infection significantly increased female fecundity by 53% (Fig. 1a). In addition, the hatch rate of deposited eggs was significantly higher in WI



**Fig. 1 | *Wolbachia* promotes fitness and ovary development in female SBPH.** **a–c** Effects of *Wolbachia* infection on fitness parameters of SBPH. **a** Egg numbers laid by a female within 15 days after emergence (Wilcoxon test,  $W = 655.5$ ,  $p = 0.0024$ ). **b** Egg hatch rate (Wilcoxon test,  $W = 671$ ,  $p = 0.0011$ ). **c** Survival curves of the adult females (Cox proportional-hazards model, hazard ratio = 2.88, 95% CI = [2.18, 3.67],  $p = 3.43 \times 10^{-5}$ ). **d** Egg numbers laid by a female through the whole life-time. Data were recorded in 2-day intervals (GLM analysis with Gaussian distribution, symbiont effect,  $df = 1$ ,  $F = 86.04$ ,  $p < 2.2 \times 10^{-16}$ ). **e** Egg numbers by a female

in the first six days after emergence (GLM analysis with Gaussian distribution, symbiont effect,  $df = 1$ ,  $F = 48.12$ ,  $p = 4.93 \times 10^{-11}$ ). The symbol  $\times$  indicates no eggs were laid at that time. **f, g** Number of mature oocytes (**f**) (GLM analysis, symbiont effect with Poisson distribution,  $df = 1$ ,  $p = 1.2 \times 10^{-10}$ ) and ovarian morphology (**g**) in female SBPH at 1-, 3-, and 5-day post-emergence. WI *Wolbachia*-infected, WU *Wolbachia*-uninfected. Data are presented as mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ . Detailed statistical information can be found in Supplementary Data 1. Source data are available in the source data file.



lines (Fig. 1b). After hatching, WI and WU planthoppers took similar times to develop from nymphs to adults. However, WI nymphs exhibited a significantly higher emergence rate (Supplementary Fig. 1), and the emerged adults lived much longer than the WU adults (Fig. 1c). We did not observe any significant differences in adult sex ratio or wing length variance (Supplementary Fig. 1).

To get more details on how *Wolbachia* affects female egg production, we counted the number of eggs laid by a female every two days until they died (Fig. 1d). WI females laid significantly more eggs in the first few days after emergence compared to WU females. They also had a longer lifespan and extended egg-laying period. Furthermore, during the first six days after emergence, WI females started laying

**Fig. 2 | *Wolbachia* upregulates the gene expression related to energy production.** **a** Workflow of collecting WI and WU SBPH whole body samples for RNA-seq analysis. The red bubble indicates the time points for sample collection. **b** *Wolbachia* density in various tissues of 3-day-old WI female adults. MG midgut, MT Malpighian tubules.  $n = 3$ . Different letters indicate significant statistical difference (ANOVA analysis with Tukey post-hoc test,  $df = 5$ ,  $F = 12.6$ ,  $p = 0.0002$ ). **c** Fluorescence in situ hybridization (FISH) on the midgut (MG), Malpighian tubules (MT), and ovary (OV) of SBPH using *Wolbachia*-specific probe (red) and the nuclear DNA stain (blue). **d** GO enrichment analysis of differentially expressed genes (DEGs)

from WI and WU body, ovary (OV), midgut (MG), and Malpighian tubules (MT). GO terms for whole-body were obtained from genes of magenta and grey60 modules generated by Weighted Gene Co-expression Network Analysis (WGCNA). GO terms for tissues were from DEGs between WI and WU samples. **e** Genes that were significantly upregulated in WI female adults across all age groups were screened ( $\log_2$  (fold change of FPKM)  $> 0.585$ , and adjusted  $p$ -value  $< 0.05$ ), with a focus on those associated with energy production. Detailed statistical information can be found in Supplementary Data 1. Source data are available in the source data file.

eggs earlier than WU females, and the numbers of eggs laid per day were significantly higher (Fig. 1e). The number of mature oocytes was also significantly higher in WI ovaries than in WU ovaries on the third and fifth days after adult emergence (Fig. 1f). This suggests that WI ovaries develop earlier and faster than WU ovaries. We also found that WI ovaries grew faster and larger than WU ovaries (Fig. 1g).

### *Wolbachia* upregulates gene expression of mitochondrion-related pathways in female SBPH

To explore the host genes regulated by *Wolbachia* in female SBPH, we performed RNA sequencing on the whole bodies of WI and WU third instar nymphs (N3), fifth instar nymphs (N5), and 1-day-old (A1dF), 3-day-old (A3dF), and 5-day-old (A5dF) adult females after emergence (Fig. 2a). After mapping the RNA-seq reads to the chromosome-level genome of SBPH<sup>22</sup>, we used the WGCNA analysis to classify SBPH genes into 26 different gene expression modules (each assigned a different color) depending on infection status, life stage and developmental time (Supplementary Fig. 2 and Supplementary Data 2). The 256 genes belonging to magenta and grey60 modules were screened out as their expression were mostly significant by both *Wolbachia* infection status and the difference between nymphs and adults. GO (Gene Ontology) enrichment analysis of these genes yielded terms related to mitochondrial outer membrane translocase complex pathway, nuclear outer membrane-endoplasmic reticulum membrane network pathway, intracellular and endoplasmic reticulum protein-containing complex pathway and endopeptidase complex pathway (Fig. 2d and Supplementary Data 2). Therefore, we hypothesize that mitochondria are involved in the physiological regulation of planthoppers by *Wolbachia*.

*Wolbachia* were found to have a higher density in the midgut and Malpighian tubules (Fig. 2b, c). Because of this and the finding that the ovaries of WI planthoppers develop faster (Fig. 1f, g), we dissected all these tissues and did RNA-seq to analyze the differential expression of planthopper genes in these tissues (Fig. 2c). We found that the number of differentially expressed genes (DEGs) in the ovaries of WI and WU females were significantly higher than those in the midgut and Malpighian tubules (Supplementary Fig. 2). Interestingly, in the GO analysis, mitochondrial outer membrane protein complexes were consistently enriched in the DEGs of multiple tissues in WI and WU females (Fig. 2d). Several pathways associated with ovarian development and energy supply like those in the ovarian fusome organization and organic acid metabolism process were significantly enriched in WI females (Supplementary Fig. 3). During the developmental stages of the females, many genes related to energy metabolism showed a significant upward trend, and among them, the genes related to energy production were significantly upregulated (Fig. 2d and Supplementary Data 3). In addition, many genes associated with energy metabolism pathways (such as carbohydrate metabolism, insect hormone biosynthesis, and insulin and mTOR signaling pathway) were highly expressed in WI females (Supplementary Data 3). These results indicated that *Wolbachia* may upregulate energy production systematically in female SBPH.

### *Wolbachia* increases ovarian development by improving the energy production

Although energy production-related genes were upregulated in WI female SBPH, the expression of mitochondrial genome-encoded genes

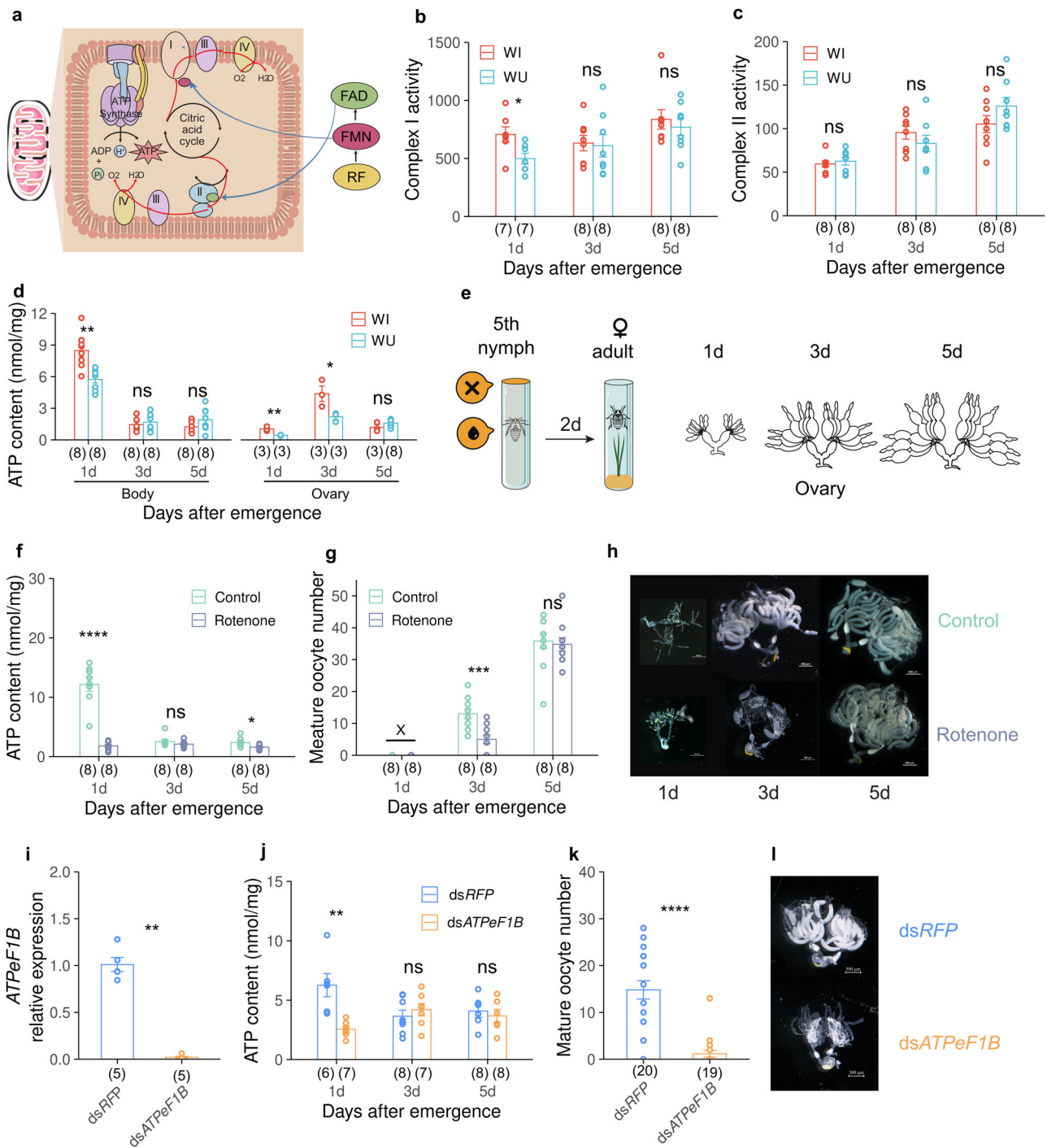
and mitochondrial density were similar between WI and WU SBPH, both in the body and ovaries (Supplementary Fig. 4a, b). We found that SBPH nuclear genes mainly associated with complexes I and II of the mitochondrial respiratory chains exhibited differential expression (Supplementary Fig. 4c–f). The involvement of mitochondrial complexes in oxidative phosphorylation is the primary source of energy production (Fig. 3a). Further analysis using Kit-based assays revealed that the mitochondrial complex I activity, rather than complex II activity, was significantly higher in 1-day-old adult WI females (Fig. 3b, c). This may explain the significantly higher ATP content in the body of 1-day-old WI females and ovaries of 1-day-old and 3-day-old WI females (Fig. 3d). However, this difference diminished later (Fig. 3d), implying a more rapid energy expenditure at the beginning.

To verify the effect of energy on ovarian development, we fed fifth instar *Wolbachia*-infected females with rotenone, a mitochondrial complex I inhibitor which affects energy production (Fig. 3e). Feeding of 5  $\mu$ M rotenone significantly reduced energy production without affecting SBPH survival or *Wolbachia* density (Supplementary Fig. 5). The ATP content was significantly higher than that of the control group at the 1 and 5 days after treatment (Fig. 3f). The number of mature oocytes in the ovaries of 3-day-old rotenone-fed SBPH females was significantly lower than that of the control group (Fig. 3g), and the oocytes developed more slowly after rotenone treatment (Fig. 3h), suggesting that the energy is essential for normal ovarian development. Additionally, we knocked down the gene expression of *ATPeF1B*, a gene important in ATP synthesis (Fig. 3i), and found that when ATP content decreased in 1-day-old females (Fig. 3j), both the ovarian development and the number of mature oocytes in WI planthoppers were significantly reduced (Fig. 3k, l). These results highlight the importance of energy production, particularly mitochondrial complex I, on ovarian development.

### *Wolbachia*-synthesized riboflavin promotes energy production

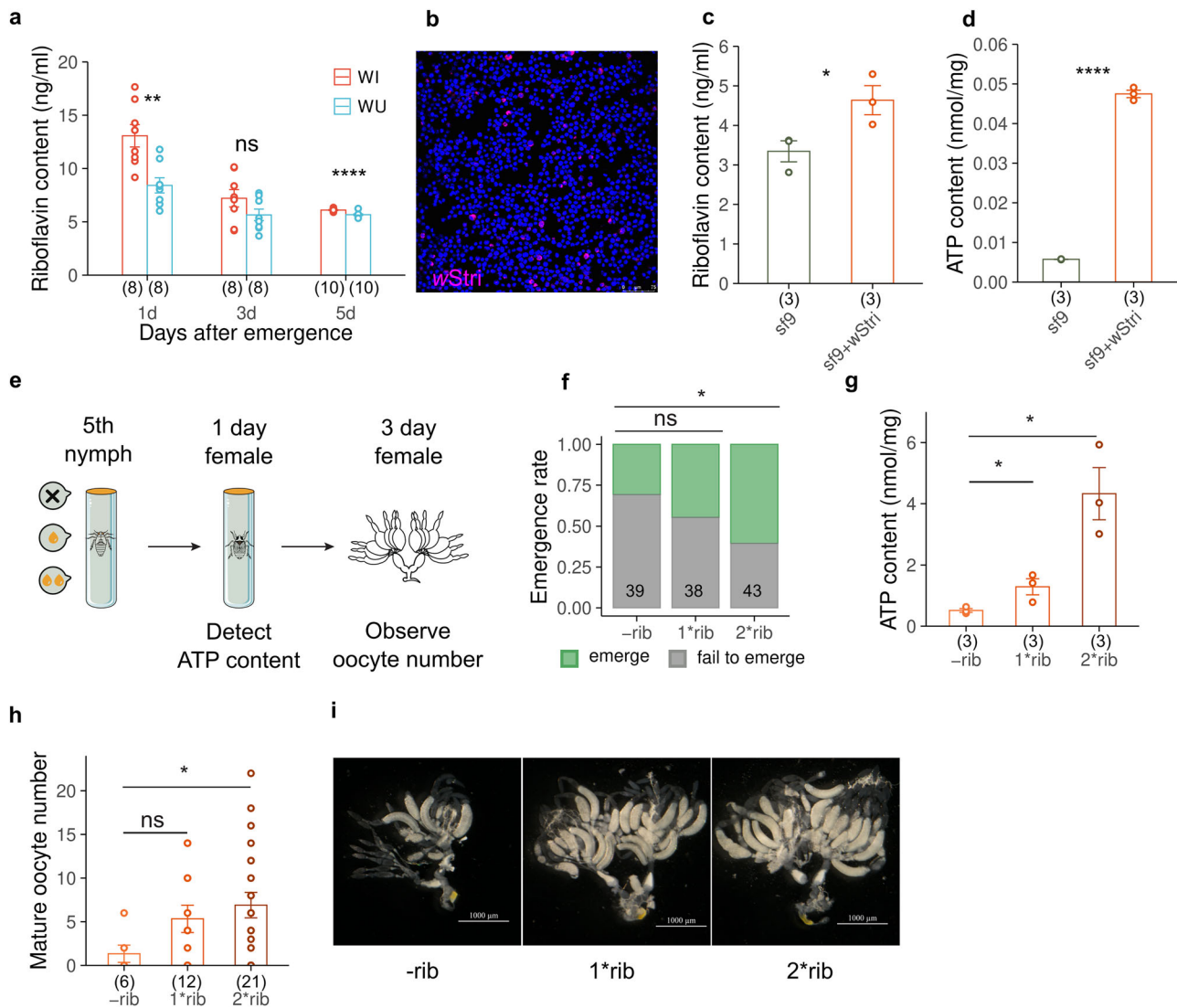
We previously reported that the genome of SBPH's *Wolbachia* (the *wStri* strain) encodes a complete set of riboflavin synthesis genes<sup>14</sup>. The riboflavin content in WI female adults was higher than the WU females, especially significantly higher in 1-day-old females (Fig. 4a). When both WI and WU SBPH were maintained on a riboflavin-deficient diet, WU females exhibited higher mortality than WI females. However, when both groups were provided with a riboflavin-supplemented diet, the survival rate of WU SBPH became comparable to that of WI individuals (Supplementary Fig. 6). Riboflavin plays an important role in energy production, serving as essential cofactors for mitochondrial complex I and complex II in the forms of FAD and FMN, respectively (Fig. 3a). To verify whether riboflavin synthesized by *Wolbachia* promotes energy production, we transferred *wStri Wolbachia* to a *Spo. dooptera frugiperda* sf9 cell line (Fig. 4b) and measured the riboflavin contents in both cell lines. The riboflavin content in the *wStri*-transfected sf9 cells was significantly higher than that of the untransfected sf9 cells (Fig. 4c), and the ATP content was eight times that of the control group (Fig. 4d). These results indicate that *Wolbachia*-synthesized riboflavin is a key factor in enhancing energy production.

We then added riboflavin to the artificial diet of WU fifth-instar nymphs and measured the ATP content and the ovarian development of the emerged female adults (Fig. 4e). Nymphs fed with riboflavin-



**Fig. 3 | *Wolbachia* upregulates SBPH females' energy production.** **a** Overview of mitochondrial oxidative phosphorylation, which includes ATP synthesis, mitochondrial respiratory chain and riboflavin (RF). The figure is redrawn based on materials from Figdraw (<https://www.figdraw.com>). **b, c** Activity of complex I (1-day samples,  $t$ -test,  $t = 2.613$ ,  $df = 12$ ,  $p = 0.0227$ ) (**b**) and complex II (**c**) in female SBPH at 1-, 3-, and 5-days post-emergence. **d** ATP content in whole body and ovaries of female SBPH.  $p = 0.0013$  for 1-day body samples ( $t$ -test,  $t = 3.995$ ,  $df = 14$ ),  $p = 0.0093$  for 1-day ovary samples ( $t$ -test,  $t = 4.707$ ,  $df = 4$ ) and  $p = 0.0482$  for 3-day ovary samples ( $t$ -test,  $t = 2.813$ ,  $df = 4$ ). WI *Wolbachia*-infected, WU *Wolbachia*-uninfected. **e** Workflow for rotenone treatment. The yellow bubble represents the addition of  $5 \mu\text{M}$  rotenone to artificial feed, while the cross symbol indicates the pure artificial diet (control). **f** ATP content in SBPH fed with rotenone or the control diet.  $p = 6.65\text{e-}07$  for 1-day samples ( $t$ -test,  $t = 8.507$ ,  $df = 14$ ) and  $p = 0.0264$  for

5-day samples ( $t$ -test,  $t = 2.482$ ,  $df = 14$ ). **g** Number of mature oocytes and **h** ovarian morphology in planthoppers fed with rotenone or control diet. The symbol  $\times$  denotes no eggs laid on day 1.  $p = 0.0009$  for 3-day samples ( $t$ -test,  $t = -3.935$ ,  $df = 18$ ). **i** Relative expression levels of *ATPeF1B* normalized to *rps5* in WI SBPH at 3 days post-dsRNA treatment ( $t$ -test,  $t = -13.01$ ,  $df = 8$ ,  $p = 1.15\text{e-}06$ ). **j** ATP content in SBPH treated *dsATPeF1B* and the *dsRFP* control.  $p = 0.0021$  for 1-day samples ( $t$ -test,  $t = -3.9853$ ,  $df = 11$ ). **k** Number of mature oocytes (Wilcoxon test,  $W = 21.5$ ,  $p = 9.6\text{e-}07$ ) and **l** ovarian morphology in planthoppers at 3 days after emergence after dsRNA treatment. Data are expressed as the mean  $\pm$  SEM. The  $t$ -test indicates Student's two-sided  $t$ -test. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; ns not significant. Detailed statistical information can be found in Supplementary Data 1. Source data are available in the source data file.

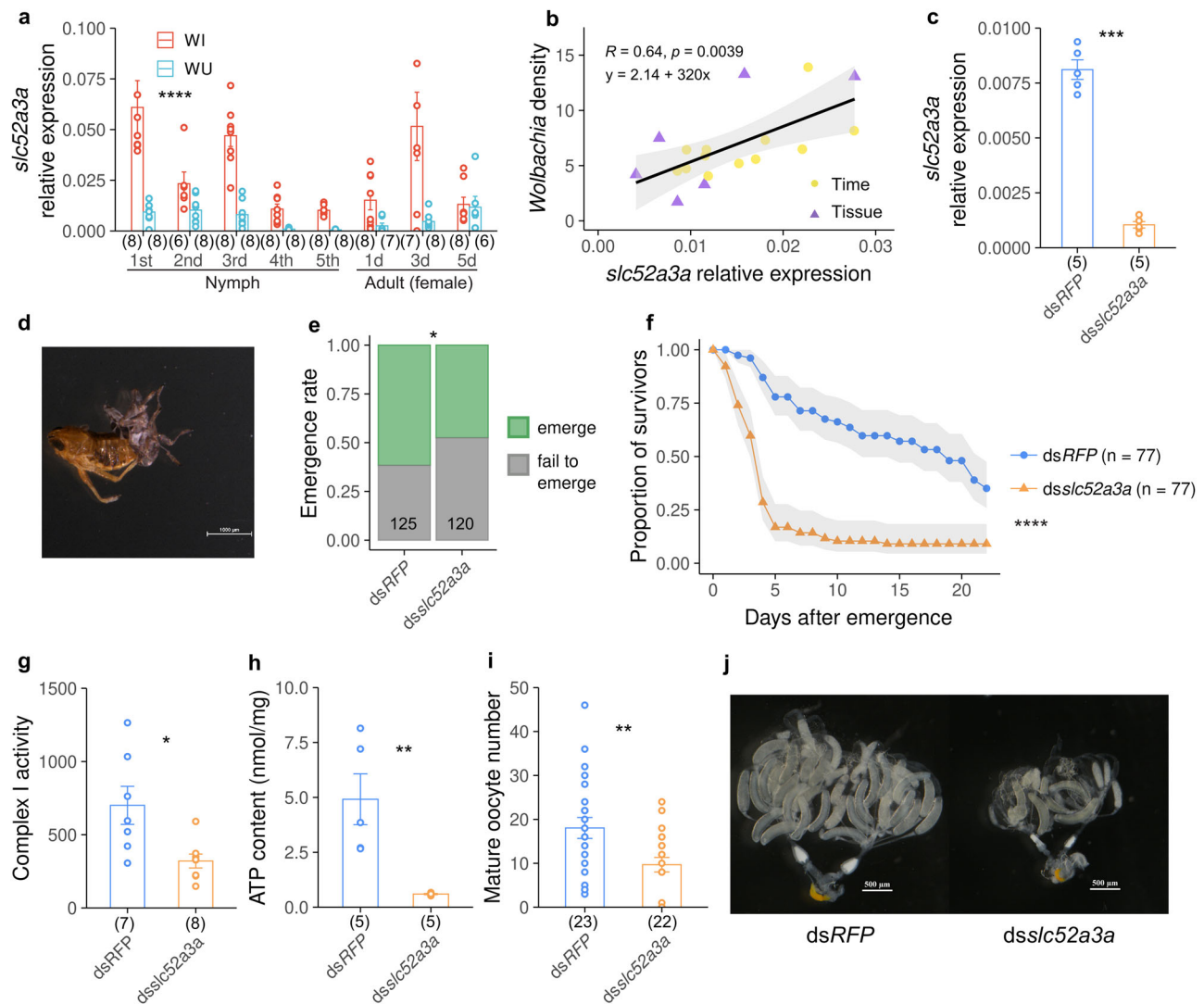


**Fig. 4 | Riboflavin synthesized by *Wolbachia* promotes the ATP-producing ability then the ovary development.** **a** Riboflavin content in female SBPH at 1-, 3-, and 5-days post-emergence.  $p = 0.0025$  for 1 day post-emergence ( $t$ -test,  $t = 3.668$ ,  $df = 14$ ),  $p = 8.095e-05$  for 5 day post-emergence ( $t$ -test,  $t = 5.063$ ,  $df = 18$ ). WI *Wolbachia*-infected, WU *Wolbachia*-uninfected. **b** FISH of *wStri*-infected *Spodoptera frugiperda* sf9 cells. Red and blue signals indicate *Wolbachia* labeled with rhodamine and DNA stained with DAPI, respectively. **c**, **d** Riboflavin content ( $t$ -test,  $t = -2.846$ ,  $df = 4$ ,  $p = 0.0466$ ) **c** and ATP content ( $t$ -test,  $t = -44.52$ ,  $df = 4$ ,  $p = 1.522e-06$ ) **d** in sf9 cells. **e** Experimental design for riboflavin treatment. Fifth instar nymphs of WU SBPH were fed riboflavin. The × symbol indicates an artificial diet lacking riboflavin (-rib), a single drop represents an artificial diet containing the normal concentration of riboflavin (1\*rib), and two drops correspond to a 2×

concentration of riboflavin (2\*rib). **f–i** Effects of riboflavin on emergence rate ( $p = 0.3033$  for comparison between -rib and 1\*rib, Chi-Squared test,  $\chi^2 = 1.060$ ,  $df = 1$ ;  $p = 0.0135$  for comparison between -rib and 2\*rib, Chi-Squared test,  $\chi^2 = 6.108$ ,  $df = 1$ ) (**f**), ATP content ( $p = 0.0457$  for comparison between -rib and 1\*rib,  $t$ -test,  $t = -2.8641$ ,  $df = 4$ ;  $p = 0.0112$  for comparison between -rib and 2\*rib,  $t$ -test,  $t = -2.8641$ ,  $df = 4$ ). (**g**), mature oocyte number ( $p = 0.1277$  for comparison between -rib and 1\*rib, Wilcoxon test,  $W = 20$ ;  $p = 0.048$  for comparison between -rib and 2\*rib, Wilcoxon test,  $W = 29.5$ ) (**h**), and ovary morphology (**i**) in SBPH. Data are presented as the mean ± SEM. The  $t$ -test indicates Student's two-sided  $t$ -test. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; ns not significant. Detailed statistical information can be found in Supplementary Data 1. Source data are available in the source data file.

containing diet had higher emergence rate (Fig. 4f), while the ATP content in the newly emerged females was significantly higher (Fig. 4g). On the third day after emergence, mature oocytes were observed in the ovaries of both groups of females fed with riboflavin, while nearly no mature oocytes were found in the ovaries of unfed females (Fig. 4h, i). However, when riboflavin was provided to WI planthoppers, this beneficial effect was no longer observed (Supplementary Fig. 7), suggesting that riboflavin is more critical for WU planthoppers. Consequently, the increase in riboflavin content enhances the energy production and ovary development of WU female planthoppers.

***Wolbachia*-upregulated riboflavin transporter *slc52a3a* is required for ovarian development**  
 Riboflavin was transported across cell membranes by the SLC52A family proteins<sup>23</sup>. We identified a SLC52A member, the *slc52a3a* gene (GenBank: ZRF44203), in the SBPH genome and found SLC52A3A is conserved across insect orders (Supplementary Fig. 8). *slc52a3a* was upregulated in WI nymphs and early female adults compared to WU SBPH (Fig. 5a). Like *Wolbachia*, *slc52a3a* was highly expressed in the midgut and Malpighian tubules and peaked on days 9 and 11 in female adults (Supplementary Fig. 9). *Wolbachia* density was found to be positively correlated with *slc52a3a* expression in various



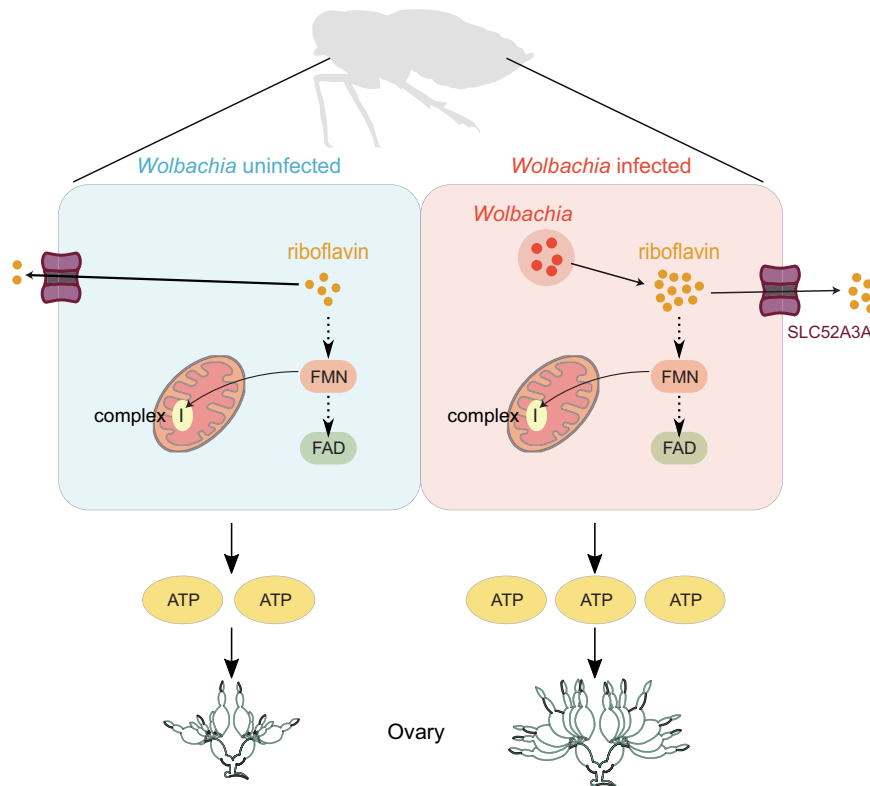
**Fig. 5 | *Wolbachia* upregulates the expression of SBPH's riboflavin transporter *slc52a3a* to promote ovary development.** **a** Relative expression of *slc52a3a* across various developmental stages in SBPH (GLM analysis with Gaussian distribution, symbiont effect,  $df = 1$ ,  $F = 59.94$ ,  $p = 6.11e-12$ ). **b** Correlation between *slc52a3a* expression and *Wolbachia* density in WI female SBPH adults. Yellow circles and purple triangles represent samples from different time points and tissues, respectively. For details, see Supplementary Fig. 9. **c** Relative expression levels of *slc52a3a* normalized to *rps5* in WI SBPH at 3 days post-dsRNA treatment ( $t$ -test,  $t = 15.06$ ,  $df = 8$ ,  $p = 3.743e-07$ ). **d**, **e** Dead planthoppers during molting of nymphs (**d**) and emergence rate (**e**) after dsRNA treatment of WI SBPH 5th instar nymphs (Chi-Squared test,  $\chi^2 = 4.36$ ,  $df = 1$ ,  $p = 0.037$ ). **f** Survival curves of emerged adults

after dsRNA treatment of 5th instar nymphs in WI SBPH (Cox model, hazard ratio = 4.68, 95%CI = [3.43, 6.38],  $p = 1.04e-13$ ). **g–j** The mitochondrial complex I activity ( $t$ -test,  $t = 2.9$ ,  $df = 13$ ,  $p = 0.0124$ ) (**g**), ATP content at 1-day female adults ( $t$ -test,  $t = 3.73$ ,  $df = 8$ ,  $p = 0.0058$ ) (**h**), mature oocyte numbers (Wilcoxon test,  $W = 368$ ,  $p = 0.0091$ ) (**i**) and ovary morphology (**j**) of 3-day-old adults after dsRNA treatment of 5th instar nymphs in WI SBPH. WI *Wolbachia*-infected, WU *Wolbachia*-uninfected. Data are presented as the mean  $\pm$  SEM for bar plots. The  $t$ -test indicates Student's two-sided  $t$ -test. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; ns not significant. Detailed statistical information can be found in Supplementary Data 1. Source data are available in the source data file.

developmental stages and adult tissues (Fig. 5b). Injecting WI 5th nymphs with dsRNA targeting *slc52a3a* significantly decreased the emergence rates, and many planthoppers died during emergence and molting (Fig. 5c–e). The females that emerged showed higher mortality, less mitochondrial complex I activity, less ATP content, and fewer mature oocytes compared to females that were not treated in the nymph stage (Fig. 5f–j). These results indicate that the promoting effect of riboflavin on host ovarian development requires *slc52a3a*. However, when *slc52a3a* was silenced in 1-day and 5-day old female adults, no significant effect was observed (Fig. S10), including 9-day and 11-day old SBPH, where *slc52a3a* showed an expression peak. This indicated that riboflavin's effect on ovarian development occurs mainly in the early post-emergence period.

## Discussion

In this study, we uncovered a mechanism by which *Wolbachia* synthesizes riboflavin, which enhances the reproductive success of planthopper-feeding planthoppers. Our findings demonstrate that riboflavin produced by *Wolbachia* promotes ovarian development and oviposition in female *Laodelphax striatellus* (SBPH). *Wolbachia* infection elevated riboflavin levels, upregulated the expression of the riboflavin transporter *slc52a3a*, and enhanced the respiratory activity of mitochondrial complex I post-emergence, leading to increased ATP production. This rise in ATP content significantly accelerated ovarian development, enabling WI females to enter the oviposition phase earlier and lay more eggs (Fig. 6). These findings provide a clearer understanding of the mechanisms underlying *Wolbachia*'s role in host



**Fig. 6 | Schematic illustration of *Wolbachia*-mediated enhancement of energy production and ovarian development in SBPH.** In *Wolbachia*-uninfected SBPH, riboflavin availability is limited due to the absence of *Wolbachia*-derived synthesis and the low expression of the riboflavin transporter *slc52a3a*. This shortage restricts mitochondrial complex I activity, potentially reducing electron transport chain function and resulting in low ATP production. As a result, ovarian development is impaired, with smaller and less mature ovaries and fewer deposited eggs. In

contrast, in the *Wolbachia*-infected SBPH, *Wolbachia* endosymbionts synthesize riboflavin, while simultaneously upregulating *slc52a3a* expression. *slc52a3a* positively correlates with *Wolbachia* density and is required for normal ovarian maturation. These combined effects increase riboflavin transportation, stimulating complex I activity and boosting ATP production. The amplified energy supply fuels ovarian development, resulting in larger, more mature ovaries and increased fecundity.

reproduction, and shed light on the intricate interplay between symbionts and their hosts.

While *Wolbachia*-mediated similar nutrient-dependent benefits have been observed in insects such as the bed bug, in which *Wolbachia*-synthesized biotin and riboflavin enhance host growth and fecundity<sup>13</sup>, the specific pathways by which these nutrients regulate reproduction remain unresolved. Our cell line experiments confirmed that *wStri* strain of *Wolbachia* actively synthesizes riboflavin, which plays a critical role in energy production and ovarian development in SBPH. In *Drosophila melanogaster*, gut microbe-synthesized riboflavin can promote host energy and ovarian development<sup>24</sup>, indicating that the gut may exert a systemic regulatory effect on the ovary through nutrition from symbiont. *Wolbachia* is located in both the female digestive tissues and the ovaries. Furthermore, the expression of riboflavin transporter *slc52a3a* was positively correlated with *Wolbachia* density. These findings, combined with RNAi results (Fig. 5f), further support the idea that riboflavin provided by *Wolbachia* plays a key role in ovarian development.

Riboflavin serves as the precursor of the cofactor FMN in mitochondrial electron transport chain complex I, which is essential for oxidative phosphorylation<sup>16</sup>. Our data show that the *wStri* strain significantly increases SBPH mitochondrial complex I activity and ATP production. This increase in mitochondrial efficiency directly correlates with accelerated ovarian maturation (Fig. 3). The discovery that *wStri* enhances ATP production through riboflavin-mediated mitochondrial activity underscores the critical role of cellular energy in supporting host reproductive processes.

Our results show that the influence of riboflavin on ovarian development is most pronounced in the early post-emergence period, which is a critical time for host reproductive success. The difference in ATP contents, complex I activity, and riboflavin content between WI and WU SBPH is most obvious in newly emerged females. Knockdown of *slc52a3a* in fifth instar nymphs significantly delayed ovarian development in adult females, while silencing *slc52a3a* in adult females did not produce the same effects. These results emphasize the dynamic nature of symbiont-derived nutrient contributions and their stage-specific impact on host biology.

In conclusion, our findings elucidate a mechanism by which *Wolbachia* enhances host reproduction through riboflavin-mediated energy production. This study advances our understanding of the intricate metabolic interplay between symbionts and their hosts, highlighting the importance of nutrients in shaping symbiotic relationships. Further studies are needed to determine whether similar mechanisms operate in other *Wolbachia*-host systems and to see if these findings can have applications in pest management and biological control strategies.

## Methods

### Insect rearing

The *Laodelphax striatellus* SBPH planthoppers were collected in rice paddies in Nanjing. Planthoppers were reared on rice seedlings and maintained in an environment chamber at 25 °C ± 1 °C, 60% relative humidity and a 16 L:8D photoperiod. The population was treated with 1 mg/ml tetracycline to obtain WU planthoppers. To produce WI

planthoppers, as well as to eliminate the effect of factors unrelated to *Wolbachia* on SBPH, *Wolbachia*-containing haemolymph was injected into nymphs of WU planthoppers as previously described<sup>14</sup>. Approximately once a month, *Wolbachia* infection status of the planthopper populations was checked by PCR amplification of the *Wolbachia* surface protein (*wsp*) gene (Supplementary Data 4).

### Biological performance assay

Fifth-instar nymphs were collected and reared in glass tubes containing several rice seedlings until emergence. The emergence rate, sex ratio, wing length type of the emerged adults, and the lifespan of female adults were recorded. Newly emerged female adults were paired with two male adults for mating. The number of eggs laid by the female, the number of hatched eggs and the times from hatching to emergence were recorded. To investigate ovarian development in female adults, ovarian samples from newly emerged individuals were dissected under a stereomicroscope. Images were captured using a stereo fluorescence microscope SMZ25 equipped with the NIS-Elements BR 4.50.00 digital imaging system (Nikon, Tokyo, Japan).

### Quantification of *Wolbachia* densities

The density of *Wolbachia* was determined using quantitative PCR (qPCR) on DNA samples extracted from SBPH as previously described<sup>14</sup>. Total DNA was isolated using the FastPure Blood/Cell/Tissue/Bacteria DNA Isolation Mini Kit (Vazyme Biotech, Nanjing, Jiangsu, China). The copy numbers of the *Wolbachia wsp* (AF020080) and the SBPH single-copy gene *actin* (AY192151) were quantified by qPCR using ChamQ SYBR qPCR Master Mix (Vazyme Biotech) on a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The relative *Wolbachia* density was calculated as the ratio of *wsp* to *actin* copy numbers. Each DNA sample was analyzed in duplicate technical replicates. The primer sequences are provided in Supplementary Data 4.

### Fluorescence in situ hybridization

The localization of *Wolbachia* in SBPH tissues was visualized using fluorescence in situ hybridization (FISH). Two rhodamine 5'-end labeled 16S rRNA *Wolbachia*-specific probes (W1: 5'-AATCCGGCC-GARCCGACCC-3' and W2: 5'-CTTCTGTGAGTACCGTCATTATC-3') were used for *Wolbachia* detection, while DAPI was employed to stain DNA. Midgut, Malpighian tubules, and ovary tissues from female SBPH were dissected and immediately fixed in 4% paraformaldehyde for 30 min. The samples were then processed according to established protocols<sup>25</sup> and imaged using a Leica TCS SP8 laser confocal microscope (Leica Wetzlar, Germany).

### Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from the samples using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and reverse-transcribed into cDNA using HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech). Gene expression levels were quantified by qPCR, as described above. Each sample included two technical replicates. Relative gene expression levels were calculated using the delta Ct method, with the *rps5* gene (XM\_039430651) as a reference control. Primer sequences are listed in Supplementary Data 4.

### Transcriptome sequencing and analysis

For the RNA-seq analyses, WI, WU nymphs in the 3rd-instar and 5th-instar and female adults of 1-day-old, 3-day-old, and 5-day-old were collected (Fig. 2A), with three biological replicates of at least 3 individuals for each stage. Additionally, the ovaries of 1-day-old females were dissected, as well as the midgut and Malpighian tubules of 3-day-old females, with three biological replicates of at least 10 individuals for each tissue. Total RNA was extracted using TRIzol. cDNA libraries were

then prepared and sequenced on a BGISEQ-500 (BGI, Shenzhen, Guangdong, China) by Beijing Genomics Institution.

The sequenced reads were cleaned using SOAPnuke v.1.5.2<sup>26</sup> and fastp v.0.20.0<sup>27</sup>. The clean reads were then mapped to the *L. striatellus* transcriptome (GCF\_014465815.1)<sup>22</sup> using STAR RNA-seq aligner v.2.7.5c<sup>28</sup>. The number of mapped reads was counted using the “-quantMode GeneCounts” parameter in STAR.

To identify differentially expressed genes (DEGs) between WI and WU samples from various tissues, gene expression was assessed using read counts with DESeq2<sup>29</sup> on R 4.4.2<sup>30</sup>. Gene expression levels were measured as Fragments per Kilobase Million (FPKM). Genes with an average FPKM greater than 5 in either treatment were considered expressed. DEGs were identified based on the criteria: average FPKM > 5, log<sub>2</sub>(fold change of FPKM) > 0.585, and adjusted *p*-value < 0.05. Transcriptomic data from WI and WU whole insects were subjected to co-expression analysis using WGCNA and hierarchical clustering based on gene connectivity dissimilarity (1-TOM)<sup>31</sup>. Gene Ontology (GO) enrichment analysis was conducted using the R package “clusterProfiler”<sup>32</sup>.

### ATP content, complex I and II activities

To measure ATP content in female SBPH, samples were homogenized in lysis buffer and centrifuged at 12,000 × *g* for 10 min to obtain the supernatant. ATP levels were quantified using an ATP assay kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's instructions.

To assess the activities of mitochondrial complexes I and II, SBPH samples were homogenized in extraction buffer on ice. The homogenate was centrifuged at 600 × *g* for 10 min at 4 °C to obtain the supernatant, which was further centrifuged at 11,000 × *g* for 15 min at 4 °C to yield the final supernatant for activity assays. The activities of complex I and complex II were evaluated using micro mitochondrial respiratory chain activity kits for complex I and II (Solarbio, Beijing, China).

ATP content and complex activities were measured using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA) by detecting chemiluminescence and absorbance at specific wavelengths, respectively. To minimize variability caused by differences in protein concentration during sample preparation, samples were normalized to the same protein concentration using a BCA Protein Assay Kit (Sangon Biotech, Shanghai, China). Each treatment included at least three biological replicates, with three individuals per replicate.

### Riboflavin content analysis

Riboflavin content was measured as previously described<sup>33</sup>. Female SBPH samples were homogenized in citrate buffer (pH = 4.5) and enzymatically digested with Taka-diastase and acid phosphatase (Sigma-Aldrich, St. Louis, MO, USA) under dark incubation (37 °C, 1 h). The hydrolysate was heat-treated (95 °C, 30 min) with periodic agitation, followed by rapid cooling and centrifugation (800 × *g*, 5 min). The supernatant was inoculated into riboflavin-dependent *Lactobacillus rhamnosus* (ATCC, 7469) cultured in Difco™ Riboflavin Assay Medium (BD, Franklin Lakes, NJ, USA). After dark incubation (37 °C, 19–24 h), absorbance at 630 nm was measured using the SpectraMax M5 microplate reader. Riboflavin concentrations were determined against a standard curve and normalized to sample weight to correct sampling bias.

### Mitochondrial complex I inhibition by rotenone

The mitochondrial complex I inhibitor rotenone (Macklin Biochemical, Shanghai, China) was dissolved in acetone to prepare a stock solution with a concentration of 1 mg/ml. Rotenone was then added to the artificial diet<sup>34</sup> at final concentrations of 0, 5, 10, and 25 μM. Fifth-instar

nymphs were fed the treated diets until emergence. The survival rate, ATP content in female adults and *Wolbachia* density were measured as described above.

### Riboflavin supplement experiment

To examine the impact of riboflavin on SBPH, the WI and WU SBPH were fed three different diets: a normal artificial diet, an artificial diet deficient in riboflavin, and a sucrose solution serving as a control. Their survival rates were recorded over a period of 15 days. In further experiments, fifth-instar nymphs were fed artificial diets supplemented with varying concentrations of riboflavin. The experiments were conducted in a rearing chamber under dark conditions. The emergence rates of WU nymphs, the ATP content in 1-day-old female adults and the number of mature oocytes in 3-day-old female adults were recorded.

### Transfection of *Wolbachia* in cell lines

Freshly laid WI eggs of SBPH (as donors) were carefully collected and immersed in 1× phosphate-buffered saline (PBS). Following the method described by Li et al.<sup>35</sup>, the *Wolbachia* was co-cultured with *Wolbachia*-free *Spodoptera frugiperda* Sf9 cells. Once the Sf9 cell line was successfully infected with *w*Stri, subsequent generations of cells were subjected for PCR confirmation to verify the presence of the *w*Stri. This process was repeated until a stably *w*Stri-infected Sf9 cell line was established. To measure the ATP and riboflavin contents, equal number of cells were sonicated after being selected out using a Countess II FL Automated Cell Counter (Thermo Fisher Scientific).

### RNA interference (RNAi)

The *slc52a3a* gene was amplified from SBPH cDNA and cloned into the pClone007 vector (Tsingke, Beijing, China). The recombinant vector served as a template for PCR amplification, and the resulting product was used to synthesize double-stranded RNA (dsRNA) with the T7 High-Yield RNA Transcription Kit (Vazyme). The dsRNA was purified following a previously described method<sup>36</sup> and adjusted to a final concentration of 5000 ng/μl. Fifth-instar nymphs or newly emerged female adults were microinjected with 250 ng of dsRNA using a Nanoliter 2010 Microinjection Pump (WPI, Sarasota, FL, USA). After the injected nymphs developed into adults, insects were collected for analyzing RNAi efficiency with RT-qPCR. The red fluorescent protein (RFP) gene was used as a control. The primer sequences are listed in Supplementary Data 4.

### Statistical analysis

Ecological data and qPCR results were first tested for normality using the Shapiro–Wilk test, followed by Student’s two-sided *t* test and ANOVA with the Tukey post-hoc test for parametric analysis or the Wilcoxon rank sum test and Kruskal–Wallis test with Bonferroni correction for non-parametric analysis. Multiple comparisons were analyzed with the “agricolae” package<sup>37</sup>. The effects of *Wolbachia* infection and time on egg numbers and oocyte numbers were analyzed using a generalized linear model (GLM). Survival curves were compared using the Cox proportional hazards model. Fisher’s exact test was used for analyzing emergence rate, wing form ratio, and sex ratio. All statistical analyses were performed in R 4.4.2<sup>30</sup>. The schematic illustrations were drawn by Inkscape 0.92 (<https://inkscape.org/>).

### Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

### Data availability

The RNA-seq data generated in this study have been deposited in the NCBI GenBank database under accession code [PRJNA1195149](https://doi.org/10.1038/s41467-025-67660-1),

[PRJNA1195150](https://doi.org/10.1038/s41467-025-67660-1), and [PRJNA1195152](https://doi.org/10.1038/s41467-025-67660-1). Source data are provided with this paper.

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## Author contributions

The authors contributed to the present study as follows: Y.D.N., X.L.B., and X.Y.H. designed the research; Y.D.N., Q.H.F., Z.H.W., M.K.W., D.S.Z., M.R.W., B.X.W., and X.L.B. performed the research and analyzed the data; Y.D.N., X.L.B., and X.Y.H. wrote and edited the manuscript; all authors read and approved the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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