

# Population Dynamics of *Wolbachia* in the Leafhopper Vector *Yamatotettix flavovittatus* (Hemiptera: Cicadellidae)

Jureemart Wangkeeree,<sup>1,4,\*</sup> Piyatida Sanit,<sup>1</sup> Jariya Roddee,<sup>2,\*</sup> and Yupa Hanboonsong<sup>3</sup>

<sup>1</sup>Department of Agricultural Technology, Faculty of Science and Technology, Thammasat University Rangsit Centre, Khlong Nueng, Khlong Luang, Pathum Thani, Thailand, <sup>2</sup>School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Suranaree, Muang, Nakhon Ratchasima, Thailand, <sup>3</sup>Department of Entomology, Faculty of Agriculture, Khon Kaen University, Nai Muang, Muang, Khon Kaen, Thailand, and <sup>4</sup>Corresponding author, e-mail: [jureemart@yahoo.com](mailto:jureemart@yahoo.com)

Subject Editor: Julie Urban

Received 8 June 2021; Editorial decision 13 October 2021

## Abstract

*Wolbachia* (Rickettsiales: Alphaproteobacteria) infections induce abnormalities in the reproductive system and affect various biological traits of the host insects. The density of *Wolbachia* is one of the major parameters that influence induced phenotypes and interactions with the hosts. *Wolbachia* occurs naturally in populations of the leafhopper *Yamatotettix flavovittatus* Matsumura (Hemiptera: Cicadellidae), which transmits phytoplasma that cause white leaf disease in sugarcane. However, the quantity and dynamics of *Wolbachia* in this leafhopper are not well understood. In the current study, we estimated the number of *Wolbachia* by absolute quantification of the copy number of *wsp*, which encodes the outer surface protein, using real-time quantitative polymerase chain reaction (PCR). This investigation was performed using natural populations and laboratory colonies from three lineages of leafhoppers (designated as UD, KK, and SK). There was no significant difference in the number of *wsp* copies in most of field-collected adults. During the immature developmental stages, there were differences in the dynamics of *Wolbachia* infection between the UD lineage and the other two lineages. However, the number of *wsp* copies increased in the early instar and plateaued in the later nymphal instars. Sex had no influence on the number of *Wolbachia* within the same lineages. The number of *Wolbachia* was relatively constant during the adult stage in the UD lineage but fluctuated in the other two lineages. In conclusion, the present data provide a framework for exploring the relationship between *Wolbachia* and the leafhopper and could facilitate future research into management strategies using *Wolbachia*.

**Key words:** *Wolbachia*, quantitative PCR, leafhopper, phytoplasma

Intracellular bacteria of the genus *Wolbachia* (Rickettsiales: Alphaproteobacteria) are reproductive manipulators that can induce abnormalities in the reproductive system of several insect hosts. These induced phenotypes include parthenogenesis, feminization, male killing, and cytoplasmic incompatibility (Werren et al. 2008, Zug and Hammerstein 2012). In addition, positive and negative effects on insect traits have been reported. For instance, *Wolbachia* infections were revealed to increase female fecundity, egg hatching, and host survival (Dobson et al. 2004, Guo et al. 2018, Bagheri et al. 2019). The deleterious effects include decrease in egg production, survival rate, and life span (Sumida et al. 2017, Lopez et al. 2018). Moreover, *Wolbachia* could reduce pathogen transmission in vector-borne diseases (Walker et al. 2011, Ye et al. 2015). Recently, *Wolbachia*-induced specific phenotypes have been proposed as a novel control strategy against insect pests (Hughes et al. 2011, Walker et al. 2011, Bourtzis et al. 2014, Hoffmann et al. 2015).

The density of *Wolbachia* has been recognized as a key factor that determines host–*Wolbachia* relationships as its titers influence the virulence or penetrance of *Wolbachia*-induced phenotypes. Moreover, it affects the efficiency of maternal transmission, which could influence successful maintenance in the host populations (Noda et al. 2001, Ikeda et al. 2003, Unckless et al. 2009, Correa and Ballard 2012, Calvitti et al. 2015). The mechanisms that regulate *Wolbachia* density within the host population are considered to result from the interactions of various factors. These include the species, developmental stage, sex, and genotype of the hosts, *Wolbachia* strain, and environmental conditions (Mouton et al. 2003, Correa and Ballard 2012, López-Madrigal and Duarte 2019). Hence, characterizing the infection density of *Wolbachia* and associated factors is essential for understanding the interplay between this bacterium and its hosts; the data generated may also help in the development of pest management strategies using *Wolbachia*.

The leafhopper *Yamatotettix flavovittatus* Matsumura (Hemiptera: Cicadellidae) is the vector of phytoplasma, a causative pathogen of the sugarcane white leaf disease (Hanboonsong et al. 2006). This disease is the most serious problem for sugarcane plantations and causes substantial yield losses in sugarcane-producing countries in Southeast Asia (Thein et al. 2012, Youichi and Hanboonsong 2017). High frequencies of *Wolbachia* infections (>80%) were reported in natural populations of *Y. flavovittatus* leafhoppers (Wangkeeree et al. 2020a). Moreover, *Wolbachia* infections drove reproductive incompatibility and showed perfect vertical transmission in *Y. flavovittatus* leafhoppers (Wangkeeree et al. 2020b). These reports highlight the possibility for future studies into leafhopper control strategies using *Wolbachia*.

As mentioned, *Wolbachia* density influences induced phenotypes as well as the efficiency of maternal transmission, leading to stable infections within the host populations. However, the number of *Wolbachia* in *Y. flavovittatus* remains to be elucidated. Therefore, the aim of the current study was to estimate the number of *Wolbachia* in three different leafhopper lineages. The number of copies of the *Wolbachia* surface protein-encoding gene (*wsp*) was determined in field-collected leafhoppers and different developmental stages of laboratory colonies.

## Materials and Methods

### Leafhopper Collection

Adult *Y. flavovittatus* were collected from sugarcane plantations in 2019 and 2020. The sugarcane fields locate in the northeastern (Udon Thani and Khon Kaen Provinces) and eastern (Sa Kaeo Province) regions of Thailand. Specimens from each location were assigned to different lineages as follows: UD, KK, and KK, representing Udon Thani, Khon Kaen, and Sa Kaeo Provinces, respectively (Supp Table S1 [online only]). Adult leafhoppers were preserved in absolute ethanol for DNA extraction, and some leafhoppers collected in 2019 were used to establish the laboratory colonies.

### Leafhopper Rearing and Specimen Sampling

Specimens collected in 2019 were used for mass rearing in a controlled laboratory; the conditions were as follows: temperature  $27 \pm 2^\circ\text{C}$ , relative humidity  $70\% \pm 5\%$ , and photoperiod 14:10 (L:D). Representative leafhoppers from each lineage were maintained in sugarcane plant cages, until the offspring emerged and then used in the experiments. To prepare the samples for quantifying number of *Wolbachia*, five pairs of male and female leafhoppers from the new generation stock were collected, released into cages containing sugarcane plants (total of six to eight cages per lineage), and allowed to mate; thereafter, the females were allowed to lay their eggs. Next, the eggs laid were collected in three phases of the embryonic stage as follows: egg-I, 2 d; egg-II, 5 d; egg-III; 9 d. Five nymphal instars (represented as N1, N2, N3, N4, and N5 for the first to fifth instars, respectively) were collected on the corresponding first days of development based on daily observations (i.e., after hatching for N1 and after molting for N2–N5). After adult emergence, males and females of similar ages were segregated and allowed to mate in the same sugarcane plant cages. Adult leafhoppers were subdivided into six stages based on their age, and sampled at ages 5, 10, 15, 20, 25, and 30 d. The specimens were preserved in absolute ethanol and stored in a freezer at  $-20^\circ\text{C}$  until use.

### DNA Extraction

Genomic DNA was extracted from individual nymphal instars and adult leafhoppers, and five specimens of eggs were pooled together.

The phenol-chloroform method was used for DNA extraction (Ausubel et al. 2008), with minor modification for leafhoppers, as described in a previous study (Wangkeeree et al. 2020a). In brief, the samples were homogenized in the extraction buffer (200 mM, Tris pH 8.0; 250 mM NaCl; 25 mM EDTA; 0.5% SDS; 0.1 mg/mL proteinase K) and overnight incubated at  $37^\circ\text{C}$ . DNA was extracted using an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), followed by centrifugation at  $10,000 \times g$  at  $4^\circ\text{C}$  for 5 min. The upper aqueous phase was moved into another tube, then extracted once with an equal volume of chloroform:isoamyl alcohol (24:1), and centrifuged at  $10,000 \times g$  at  $4^\circ\text{C}$  for 5 min. DNA pellets were obtained by precipitation with sodium acetate and isopropanol centrifugation at  $10,000 \times g$  at  $4^\circ\text{C}$  for 20 min, and the supernatants were removed. DNA pellets were washed with 70% absolute ethanol, air-dried, and resuspended in 20  $\mu\text{l}$  of TE buffer (10 mM Tris, 1 mM EDTA). DNA concentration was measured in the extracts using a Nanodrop spectrophotometer (NanoDrop Lite; Thermo Scientific) before storage at  $-20^\circ\text{C}$  until use for analysis.

### Wolbachia Detection

To confirm the infection status, field-collected leafhoppers were tested for the prevalence of *Wolbachia* using polymerase chain reaction (PCR) with specific primers. The *wsp* gene was amplified using the forward primer 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and the reverse primer 961R (5'-AAAAATTAACGCTACTCCA-3') (Zhou et al. 1998), which generated a 610 base-pair product. The PCR procedure for *Wolbachia* detection in the leafhoppers followed the method of Wangkeeree et al. (2020a). In brief, the reactions were performed with 25- $\mu\text{l}$  reaction mixtures comprising the following components: 2  $\mu\text{l}$  DNA template, 1 $\times$  reaction buffer, 2.5 mM MgCl<sub>2</sub>, 0.5  $\mu\text{M}$  primers, 0.2 mM dNTPs, and 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The cycling conditions were as follows: initial denaturation ( $94^\circ\text{C}$ , 5 min); 30 cycles of denaturation at  $95^\circ\text{C}$  (1 min), annealing at  $55^\circ\text{C}$  (1 min), extension at  $72^\circ\text{C}$  (1 min); and final extension at  $72^\circ\text{C}$  (10 min). The PCR products were visualized on 1% agarose gels, and the bands were stained with SYBR Safe DNA Gel Stain (Invitrogen).

### Construction of qPCR Standard Curves

The specific primers were designed based on the *wsp* gene sequences from the three lineages of *Y. flavovittatus* (UD, KK, and KK lineages) and deposited at the GenBank database under accession numbers MT949318–MT949347. The primers were designed and checked for specificity using the Primer-BLAST program of NCBI. The forward primer *wYfla-F* (5'-GGTGTGGTGCAGCGTATGT-3') and the reverse primer *wYfla-R* (5'-TCCGCCATCATCTTTAGCTGT-3') were used to amplify a 198-bp fragment of *wsp*. The fragments were amplified by PCR as described above (except annealing and extension for 30 s for each). The PCR products were visualized on a 1% agarose gel and the bands were stained with SYBR Safe DNA Gel Stain (Invitrogen). The *wsp* amplicons were ligated to plasmid the pCR4-TOPO TA vector, and then transformed into TOP10 Competent Cells (TOPO-TA Cloning kit; Life Technologies, Carlsbad, CA), according to the manufacturer's procedures. Recombinant plasmids were purified using the Purelink Quick Plasmid Miniprep Kit (Life Technologies).

The plasmids containing the insert (size 4154 bp including plasmid vector 3956 bp and insert 198 bp) were linearized with PstI. The digested plasmids were purified and quantified using the Nanodrop spectrophotometer and copy numbers of *wsp* fragments were calculated as the formula (Whelan et al. 2003):

$$\text{Number of copies}/\mu\text{l} = \frac{6.022 \times 10^{23} \text{ (molecules/mole)} \times \text{DNA concentration (g}/\mu\text{l)}}{\text{Size of the clone in bp} \times 660 \text{ Daltons}}$$

The standard curve was generated using this linearized plasmid containing the *wsp* sequence, which was subjected to five serial dilutions ( $10^7$ – $10^3$  copies).

### Quantitative Real-time PCR

Specimens used to quantify the copy number of *wsp* included individual adult males and females from natural populations of three lineages ( $n = 5$  adults for each sex/lineage), groups of five eggs ( $n = 5$  pooled eggs for each embryonic stage/lineage), individual nymphs from the first to fifth instar ( $n = 5$  nymphs for each stage/lineage), and individual adult males and females ( $n = 5$  adults for each sex/ages/lineage). The quantity of genomic DNA in the specimens was measured using a spectrophotometer and diluted to a concentration of 50 ng/ $\mu\text{l}$  before analysis. qPCR was carried out using the Applied Biosystems StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA). Absolute quantification was performed according to the methods of Dossi et al. (2014), with minor modifications. In brief, reactions were performed in 20  $\mu\text{l}$  final volume which consisted of the qPCR components were as follows: 1  $\mu\text{l}$  (final 50 ng) template DNA, 0.5  $\mu\text{l}$  (0.5  $\mu\text{M}$ ) of primers (*wYfla-F* and *wYfla-R*), and 10  $\mu\text{l}$  of SYBR Green Master Mix (Applied Biosystems). The cycling conditions were as follows: 95°C for 5 min, followed by 30 cycles at 95°C for 45 s, 55°C for 30 s, and 60°C for 30 s. The samples and serial dilutions of standards were distributed in triplicate wells. The reaction mixtures without DNA were used in all amplifications as negative controls. The copy number of *wsp* in the unknown samples was quantified by comparing the Cq values (cycle threshold) against those produced by serial dilutions of standards. The absolute copy number of *wsp* was determined to estimate the number of *Wolbachia* per unit concentration of the leafhopper DNA. The qPCR efficacy was >90% and  $R^2$  was >99% for all assays (Supp Fig. S1 [online only]). A melting curve analysis was performed under the conditions of 60–95°C, and showed that one correct peak was produced for all tests (Supp Fig. S2 [online only]).

### Statistical Analysis

We checked whether the data obtained using the Kolmogorov–Smirnov test were normalized prior to using the parametric methods of statistical analysis. The number of copies of *wsp* from *Wolbachia* from different geographical locations and different ages from each stage was determined. Statistically significant differences were determined using the one-way analysis of variance and comparisons of the means was performed using Tukey's honest significant difference test (Tukey's HSD). Number of copies of *wsp* in male and female leafhoppers was compared using the *t*-test. All statistical analyses were performed using IBM SPSS Statistics 20.

## Results

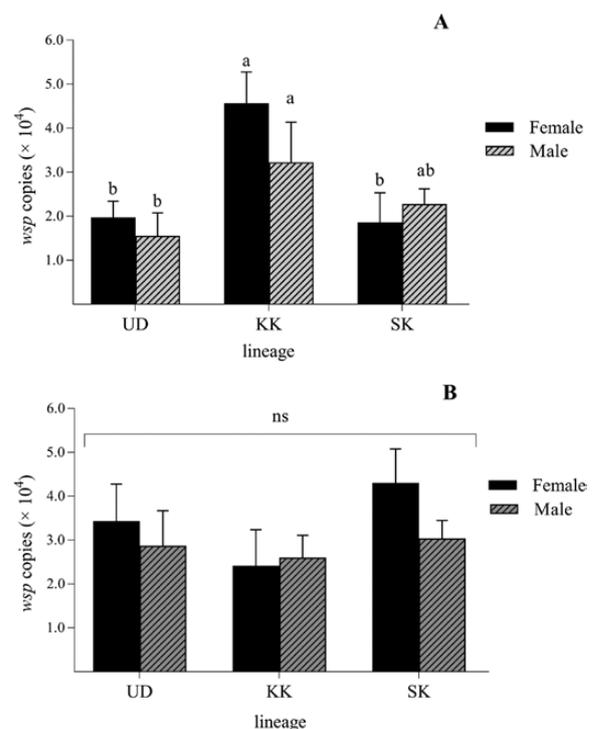
### Infection Incidence and Number of *Wolbachia* in Natural Populations

Infection statuses and number of *Wolbachia* were determined in three lineages of *Y. flavovittatus* (designated as UD, KK, and SK for leafhoppers that were collected from Udon Thani, Khon Kaen, and Sa Kaeo Provinces, respectively). High frequencies of *Wolbachia* infections were detected in all populations from both collection years, with a positivity rate of 83–100% among the individuals tested (Supp Table S1 [online only]). Meanwhile, the qPCR

results of the leafhopper that were collected in 2019 revealed that the number of *wsp* copies ranged from  $1.55 \times 10^4$  to  $4.56 \times 10^4$  copies. No significant difference was observed in most of the leafhoppers across lineages and between sexes. One exception was that females of the KK lineage showed the highest number of *wsp* copies, which was significantly different from that of other samples ( $F_{(5,29)} = 4.31$ ,  $P = 0.006$ ; Fig. 1A). Similarly, no significant difference in the number of *wsp* copies was detected among different lineages and between sexes of leafhoppers that were collected in 2020 ( $F_{(5,29)} = 0.96$ ,  $P = 0.458$ ). The mean number of *wsp* copies ranged from  $2.41 \times 10^4$  to  $4.30 \times 10^4$  copies (Fig. 1B).

### Number of *Wolbachia* in the Immature Developmental Stage

*Wolbachia* amount was also determined in *Y. flavovittatus* leafhoppers that were collected from different environments and reared in the laboratory under similar conditions. The number of *wsp* copies was compared within each stage during the immature developmental periods. In the embryonic stage of the UD lineage, the number of *wsp* copies increased with successive stages; the copy number in 9-d-old eggs was significantly higher than that in 2- and 5-d-old eggs ( $F_{(2,14)} = 6.45$ ,  $P = 0.036$ ). Significant difference was also found in the nymphal stages of this lineage ( $F_{(4,24)} = 11.44$ ,  $P < 0.001$ ). The lowest number of *wsp* copies was found in the first instar ( $1.02 \times 10^4$  copies), and then it gradually increased during the second and third nymphal instars. The copy number further increased in the later nymphal instars, with the highest copy in the last two instars (fourth and fifth nymphal stage), with  $4.02 \times 10^4$  and  $3.94 \times 10^4$  copies, respectively (Fig. 2).



**Fig. 1.** Number of *Wolbachia* in field-collected *Y. flavovittatus* leafhoppers of three different lineages. Specimens collected in (A) 2019 and (B) 2020. Values represent the mean number ( $\pm$ SE) of *wsp* gene copies per 50 ng of host genomic DNA ( $n = 5$ ). Different letters above bars indicate a significant difference determined by Tukey's HSD test, ns; no significant difference (year 2019;  $F_{(5,29)} = 4.3$ ,  $P = 0.006$ , year 2020; ( $F_{(5,29)} = 0.96$ ,  $P = 0.458$ ). KK, Khon Kaen; SK, Sa Kaeo; UD, Udon Thani.

Similar trends were observed in the KK and SK lineages, in which no significant difference in the number of *wsp* copies was found in the embryonic stage (KK lineage;  $F_{(2,14)} = 1.45$ ,  $P = 0.132$ , SK lineage;  $F_{(2,14)} = 2.05$ ,  $P = 0.337$ ). The mean of *wsp* copies ranged from  $0.43 \times 10^4$  to  $0.60 \times 10^3$  copies in the KK lineage and from  $0.29 \times 10^3$  to  $0.63 \times 10^3$  copies in the SK lineage (Figs. 3 and 4). In the nymphal stage of the KK lineage, the number of *wsp* copies was low in the first nymphal instar ( $1.85 \times 10^4$  copies), with significant differences compared with that in other nymphal instars ( $F_{(4,24)} = 1.92$ ,  $P = 0.018$ ). Thereafter, it increased in the second instar and remained stable until the fifth instar. The average amount in these stages ranged from  $3.27 \times 10^4$  to  $3.64 \times 10^4$  copies, with no significant differences among the second through fifth instars (Fig. 3). The same trend was found in the SK lineage; the mean *wsp* copies in the first instar were significantly lower ( $2.15 \times 10^4$  copies) than in the latter instars ( $F_{(4,24)} = 0.94$ ,  $P = 0.031$ ). The number of *wsp* increased at the second instar and remained stable until the last instar of the nymph. The mean *wsp* copies ranged from  $2.15 \times 10^4$  to  $3.64 \times 10^4$  copies, with no significant differences among the second through fifth instar (Fig. 4).

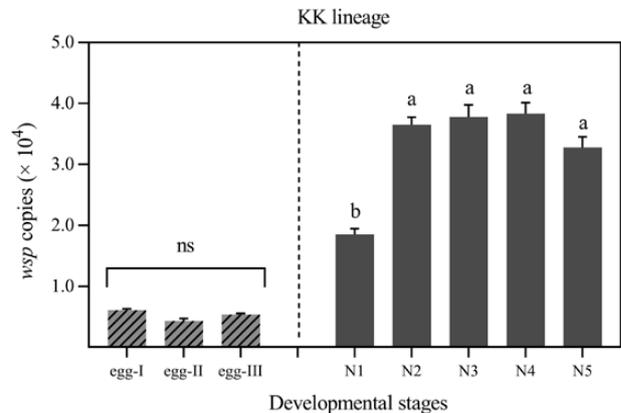
Moreover, the mean *wsp* copies in the same growth stage were compared among different leafhopper lineages. No significant difference was found in 2- and 5-d-old egg stages. In contrast, the amount of *wsp* in 9-d-old eggs of the UD lineage was significantly higher than that in the KK and SK lineages ( $F_{(2,14)} = 51.44$ ,  $P < 0.001$ ; Supp Table S2 [online only]). For nymphal development, significant differences among lineages were observed only during the first- and second-instar stages, with the mean copy number of *wsp* being lower in the UD lineage than in the KK and SK lineages (first instar;  $F_{(2,14)} = 4.02$ ,  $P = 0.049$ , second instar;  $F_{(2,14)} = 5.741$ ,  $P = 0.02$ ; Supp Table S2 [online only]). No statistically significant difference was detected in the third to fifth nymphal instar stages (Supp Table S2 [online only]).

### Number of *Wolbachia* in the Adult Developmental Stages

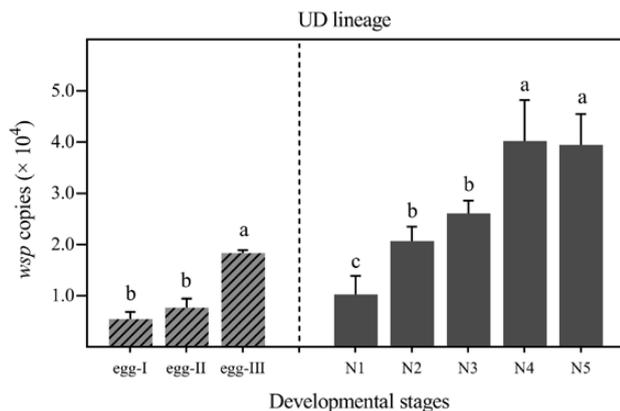
The number of *wsp* copies was also investigated during the adult developmental stages of female and male leafhoppers. For each lineage, the *wsp* copies were compared among different ages within the same

sexes. qPCR results revealed that the *wsp* copies in the UD lineage ranged from  $7.11 \times 10^4$  to  $9.88 \times 10^4$  copies for females and  $4.81 \times 10^4$  to  $9.10 \times 10^4$  for male leafhoppers (Fig. 5). Within this lineage, the number of *wsp* copies was constant across ages and similar between the sexes. No significant difference was observed over the entire life span of female and male leafhoppers (female;  $F_{(5,29)} = 0.60$ ,  $P = 0.695$ , male;  $F_{(5,29)} = 1.10$ ,  $P = 0.387$ ). However, in both sexes, the highest *wsp* copies were observed at 25 d of age, whereas the lowest copy number was detected at 30 d of age (Fig. 5).

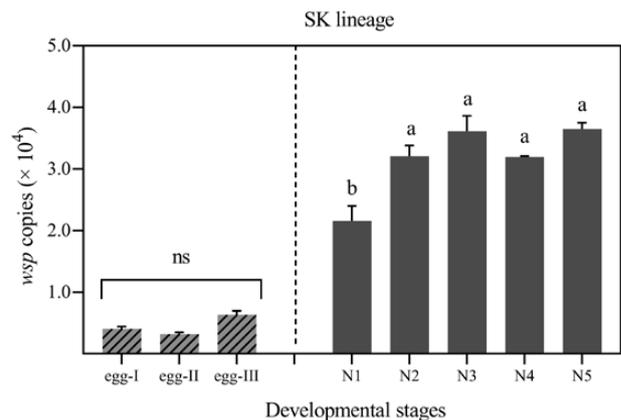
Furthermore, the trends of *wsp* copies in adult leafhoppers from the KK and SK lineages were quite similar. The number of *Wolbachia* fluctuated throughout the development of the adult stage, and these dynamics were quite similar for both sexes (Figs. 6 and 7). In the KK lineage, no significant difference in the number of *wsp* copies



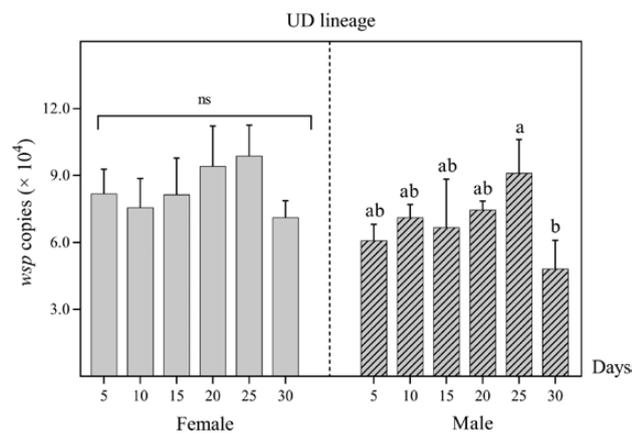
**Fig. 3.** Number of *Wolbachia* in the immature developmental stages of *Y. flavovittatus* leafhoppers of the Khon Kaen (KK) lineage. Egg-I to Egg-III refer to 2, 5, and 9 d after egg laying; N1 to N5 refer to the first to fifth instar of the nymphal stages. Values represent the mean number ( $\pm$ SE) of *wsp* gene copies per 50 ng of host genomic DNA ( $n = 5$ ). Different letters above bars indicate a significant difference determined within the same stage by Tukey's HSD test; ns, no significant difference (egg;  $F_{(2,14)} = 1.45$ ,  $P = 0.132$ , nymph;  $F_{(4,24)} = 1.92$ ,  $P = 0.018$ ).



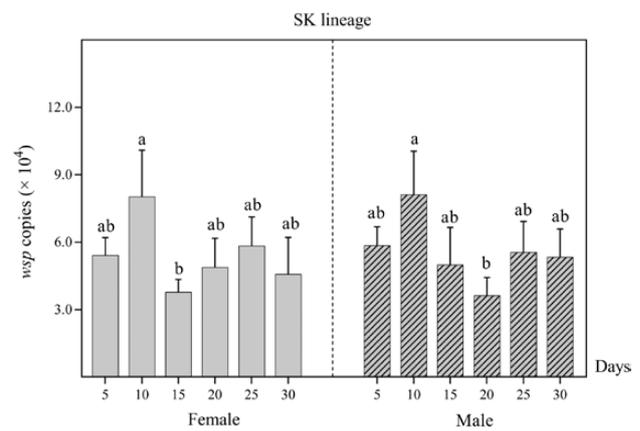
**Fig. 2.** Number of *Wolbachia* in the immature developmental stages of *Y. flavovittatus* leafhoppers of the Udon Thani (UD) lineage. Egg-I to Egg-III refer to 2, 5, and 9 d after egg laying; N1 to N5 refer to the first to fifth instar of the nymphal stages. Values represent the mean number ( $\pm$ SE) of *wsp* gene copies per 50 ng of host genomic DNA ( $n = 5$ ). Different letters above bars indicate a significant difference determined within the same stage by Tukey's HSD test (egg;  $F_{(2,14)} = 6.45$ ,  $P = 0.036$ , nymph;  $F_{(4,24)} = 11.44$ ,  $P < 0.001$ ).



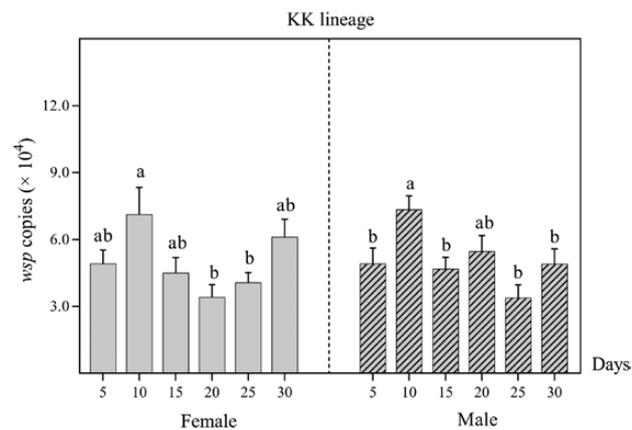
**Fig. 4.** Number of *Wolbachia* in the immature developmental stages of *Y. flavovittatus* leafhoppers of the Sa Kaeo (SK) lineage. Egg-I to Egg-III refer to 2, 5, and 9 d after egg laying; N1 to N5 refer to the first to fifth instar of the nymphal stages. Values are the mean number ( $\pm$ SE) of *wsp* gene copies per 50 ng of host genomic DNA ( $n = 5$ ). Different letters above bars indicate a significant difference determined within the same stage by Tukey's HSD test; ns, no significant difference (egg;  $F_{(2,14)} = 2.05$ ,  $P = 0.337$ , nymph;  $F_{(4,24)} = 0.94$ ,  $P = 0.031$ ).



**Fig. 5.** Number of *Wolbachia* during the adult developmental stages of *Y. flavovittatus* leafhoppers of the Udon Thani (UD) lineage. Values represent the mean number ( $\pm$ SE) of *wsp* gene copies per 50 ng of host genomic DNA ( $n = 5$ ). Different letters above bars indicate a significant difference determined within the same sex by Tukey's HSD test (female;  $F_{(5,29)} = 0.60$ ,  $P = 0.695$ , male;  $F_{(5,29)} = 1.10$ ,  $P = 0.387$ ), ns; no significant difference.



**Fig. 7.** Number of *Wolbachia* during the adult developmental stages of *Y. flavovittatus* leafhoppers of the Sa Kaeo (SK) lineage. Values represent the mean number ( $\pm$ SE) of *wsp* gene copies per 50 ng of host genomic DNA ( $n = 5$ ). Different letters above bars indicate a significant difference determined within the same sex by Tukey's HSD test (female;  $F_{(5,29)} = 1.18$ ,  $P = 0.357$ , male;  $F_{(5,29)} = 1.22$ ,  $P = 0.334$ ).



**Fig. 6.** Number of *Wolbachia* during the adult developmental stages of *Y. flavovittatus* leafhoppers of the Khon Kaen (KK) lineage. Values represent the mean number ( $\pm$ SE) of *wsp* gene copies per 50 ng of host genomic DNA ( $n = 5$ ). Different letters above bars indicate a significant difference determined within the same sex by Tukey's HSD test (female;  $F_{(5,29)} = 2.59$ ,  $P = 0.053$ , male;  $F_{(5,29)} = 3.41$ ,  $P = 0.019$ ).

was detected for female leafhoppers, which ranged from  $3.41 \times 10^4$  to  $7.12 \times 10^4$  copies ( $F_{(5,29)} = 2.59$ ,  $P = 0.053$ ). However, for male leafhoppers, the number ranged from  $3.38 \times 10^4$  to  $7.34 \times 10^4$  copies ( $F_{(5,29)} = 3.41$ ,  $P = 0.019$ ; Fig. 6). The trend of fluctuation throughout the adult stages was observed in the SK lineage, but no significant difference in the number of *wsp* copies was observed between the sexes (female;  $F_{(5,29)} = 1.18$ ,  $P = 0.357$ , male;  $F_{(5,29)} = 1.22$ ,  $P = 0.334$ ). The mean *wsp* copies ranged from  $3.77 \times 10^4$  to  $8.03 \times 10^4$  copies and from  $3.62 \times 10^4$  to  $8.12 \times 10^4$  copies for female and male leafhoppers, respectively (Fig. 7).

Moreover, for each lineage, the number of *wsp* copies between female and male leafhoppers at the same age was compared, and no significant difference was observed (Supp Tables S3–S5 [online only]). Notably, the average number of *wsp* copies was higher than  $4.56 \times 10^4$  in most adults from laboratory colonies, which was the highest copy number found in field-collected females of the KK lineage.

## Discussion

Investigations of *Wolbachia* quantity have implications in understanding the relationships between this bacterium and its hosts. To evaluate the number of *Wolbachia*, we chose the *wsp* gene, which encodes a *Wolbachia* surface protein and is generally used to quantify the *Wolbachia* using qPCR. Based on a single-copy gene present in each genome, the absolute copy number of *wsp* was determined to estimate the number of *Wolbachia*. This method has been used to quantify *Wolbachia* in several insect hosts (Zhang et al. 2010, Zhou and Li 2016, Hosseinzadeh et al. 2019, Lu et al. 2019). To our knowledge, this is the first study to describe number of *Wolbachia* in each developmental stage of *Y. flavovittatus* and we found that the copy number varied among different leafhopper lineages.

The findings of the present study indicate that no significant difference in the number of *Wolbachia* in most of field-collected leafhopper across lineages and between sexes, with occasional variations in females of the KK lineage in 2019. The factors that may influence *Wolbachia* density in natural populations of hosts originating from different geographical locations include insect genotype and *Wolbachia* strain (Unckless et al. 2009, Hoffmann et al. 2014). Moreover, environmental conditions could be one of the major factors that influence *Wolbachia* density (Mouton et al. 2007). *Wolbachia* density in the psyllid *Diaphoria citri* (Kuwayama) was found to be different among geographical populations (Chu et al. 2016), and *Wolbachia* density in the leafhoppers and planthoppers exhibits variations across different populations (Wiwatanaratnabutr 2015). However, additional investigations are required to determine the influence of environmental changes on the number of *Wolbachia* in *Y. flavovittatus* leafhoppers. In addition, we suggest that some variations in the number of *Wolbachia* in this study may also be influenced by the age of the specimens collected from the fields. Similarly, we observed fluctuations in the number of *Wolbachia* throughout the adult developmental stage of the laboratory colony.

We also investigated the number of *Wolbachia* in different developmental stages of three lineages reared in the laboratory under the same conditions. During the embryonic stage of these three leafhopper lineages, there were differences in the dynamics of *Wolbachia* infection between the UD lineage and the other two lineages. This

may be due to the specific response of each developmental stage to the difference in the genotype of *Y. flavovittatus* and *Wolbachia* strain.

In the present study, the absolute copy number of *wsp* was assessed without an internal control of the host gene. To avoid the inaccuracy in data, the quantity of *Wolbachia* was not compared between the embryonic and nymphal stages of *Y. flavovittatus*. However, a difference in the number of *Wolbachia* between the egg and nymphal stages has been reported. In the psyllid *D. citri*, *Wolbachia* density increased across developmental stages, with lower levels during embryonic development and gradually increasing across the nymph stages (Dossi et al. 2014). This is not surprising as host physiology and tissues vary significantly during growth and development. Therefore, the physiology of the host may influence the density of *Wolbachia* during development, directly by restrictions imposed on the space available for *Wolbachia* replication in the cells or tissues they occupy. However, high *Wolbachia* titers in the embryonic stage compared with those in the first-instar nymph were reported in the psyllid *D. citri*. The reduction in the number of *Wolbachia* after egg hatching might be due to its inability to resist the immune response in nymphal tissues (Ren et al. 2018).

During nymphal development, in all three leafhopper lineages, the number of *wsp* copies increased in the first or second nymph instars and appeared to be relatively constant thereafter until the last instar. This is in contrast with previous findings for the psyllid *D. citri*, in which *Wolbachia* density increased in successive nymphal instars (Ren et al. 2018), and in the beetle *Tribolium confusum* (Jacquelin du Val), in which *Wolbachia* density increased with larval development from the first to the last instar (Lu et al. 2019). The continuous growth of *Wolbachia* across developmental stages could be explained by the fact that the density dynamics are tightly modulated by host ontogeny, such that the proliferation of *Wolbachia* depends on living cells in their hosts (Ruang-areerate et al. 2004). Nevertheless, the number of *Wolbachia* in *Y. flavovittatus* leafhoppers increased in the early nymphal stages and was relatively stable from the third to the last nymphal stages. We speculate that this saturation of growth may be attributed to the limitation of space available in the nymphal body. As previously reported, in nymphs and adults of *Y. flavovittatus* leafhoppers, *Wolbachia* localized and concentrated in bacteriomes (Wangkeeree et al. 2020a); thus, *Wolbachia* replication is restricted by the space available in the cells or tissues they invaded.

For the adult stages of *Y. flavovittatus*, consistent result was obtained in both sexes from three lineages, in which the average number of *Wolbachia* did not increase with aging. In contrast, in the case of the psyllid *D. citri*, *Wolbachia* density continued to increase with aging in females (Dossi et al. 2014), and a similar trend was also reported in the wasp *Habrobracon hebetor* Say (Bagheri et al. 2019). We suggest that space restriction may be due to the localization of *Wolbachia* in the bacteriome of *Y. flavovittatus* leafhoppers, as mentioned above.

The number of *Wolbachia* in *Y. flavovittatus* adult was not compared with that in the egg and nymphal stages. Variations in *Wolbachia* density across developmental stages have been reported in other insects, for instance, *Wolbachia* density in adults of the beetle *Octodonta nipae* (Maulik) was higher than that in the immature stages (Ali et al. 2018). This correlation between *Wolbachia* density and developmental stages has also been reported in the wasp *H. hebetor* (Bagheri et al. 2019) and the beetle *Paederus fuscipes* Curtis (Ge et al. 2020). To improve the accuracy of comparison of *Wolbachia* density among different developmental stage or tissues, future studies using relative qPCR to quantify the internal control gene in *Y. flavovittatus* are necessary.

The number of *wsp* copies in *Y. flavovittatus* adults varied across lineages. The UD lineage had a relatively constant number copy compared with the other two lineages (KK and SK), in which the *wsp* copies fluctuated throughout adult development. Moreover, we found that leafhoppers of the UD lineage exhibited a higher number of *wsp* copies at most of ages than their KK and SK lineage counterparts. This finding is consistent with reports for the brown planthopper *Nilaparvata lugens* (Stål), in which there is a fluctuation in *Wolbachia* density during the adult stages across planthopper lineages (Zhang et al. 2010). Differences in *Wolbachia* density are known to result from interactions between the genotype of the hosts and that of *Wolbachia* strain (Mouton et al. 2007). Thus, the difference in the number of *wsp* copies among lineages may have been contributed by the genotype of *Y. flavovittatus* originating from different geographical locations, as well as *Wolbachia* strain associated with each lineage.

Regarding *Wolbachia* strain, based on the *wsp* gene, there was a single supergroup B in the UD, KK, and SK lineages. However, based on nucleotide sequences of housekeeping genes (*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*), allelic profiles comprise different sequence typing among these three lineages (Wangkeeree et al. 2021). This may be a factor responsible for the differences observed among each lineage. Thus, we hypothesize that in leafhoppers of the UD lineage, *Wolbachia* could have adapted to the leafhopper tissues by avoiding the host immune response, as evidenced by the higher and relatively constant levels in the adult stages. The fluctuation in the number of *Wolbachia* in the KK and SK lineages might be a response to several factors during this developmental period. These may include tissue tropism and immune system of the hosts (López-Madriral and Duarte 2019), expression of the host gene that regulates *Wolbachia* proliferation (Serbus et al. 2011), as well as the antagonist microorganisms within the same host (Goto et al. 2006). However, the underlying mechanisms of fluctuation in number of *Wolbachia* in adult *Y. flavovittatus* should be further studied.

We observed no significant differences between sexes, suggesting that the number of *Wolbachia* is not regulated differently in the two sexes of *Y. flavovittatus*. Similarly, no significant difference in *Wolbachia* density was detected in males and females of the beetle *O. nipae* (Ali et al. 2018). Variation in *Wolbachia* density between sexes has been reported; for instance, *Wolbachia* density in the females of the fly *Drosophila simulans* and the mosquito *Aedes albopictus* (Skuse) was higher than that in the males (Tortosa et al. 2010, Correa and Ballard 2012). In contrast, the infection level of *Wolbachia* in the males of the psyllid *D. citri* was higher than that in the females (Hoffmann et al. 2014, Ren et al. 2018). These differences could be explained by mechanisms that drive appropriate ranges of infection burden by *Wolbachia* within hosts; however, these mechanisms seem highly dependent on the *Wolbachia*-host and sex-specific interactions.

A possible limitation of this study is that we performed absolute quantification of *wsp* copies in the different life stages of the leafhopper. Relative quantification, in which the ratio of the amount of target gene and the amount of internal control gene is measured, may be more accurate. However, good correspondence has been found between the relative and absolute quantifications to determine *Wolbachia* density (Zhou and Li 2016). Therefore, our method of quantification is unlikely to substantially skew the results. To improve the accuracy of measurements, suitable internal control genes should be screened for further studies in *Y. flavovittatus*. Nevertheless, suitable internal control genes should be screened for further studies in *Y. flavovittatus*. In addition, another approach to improve the accuracy of measurements is droplet digital PCR, which

is used for the quantification of *Wolbachia* in insect hosts (Fisher et al. 2019). Apart from primers, specific probes are also used in the reaction to assess *Wolbachia* density. This technique should be further developed for *Y. flavovittatus* leafhoppers.

In summary, our findings suggest that the dynamics of *Wolbachia* infection varied among different leafhopper lineages. Within the same lineages, the number of *Wolbachia* was not influenced by sexes. However, in adult stages, it was relatively constant in the UD lineage but fluctuated in the KK, and SK lineages. Previous studies have revealed the perfect vertical transmission of *Wolbachia* and reproductive incompatibility in the UD, KK, and SK leafhopper lineages. These studies highlight the possibility for future investigations on control strategies using *Wolbachia*. In this context, *Wolbachia* may have a novel use as a vehicle for transgenes into the leafhopper vector populations (Xi et al. 2005) and/or use in artificial transinfection methods (Gong et al. 2020). In the present study, we elucidated the number of *Wolbachia* associated with each leafhopper lineage. These could be useful information for future development of transinfection, that is, infected-leafhoppers of the UD lineage may be an appropriate source as donors as this lineage exhibited a relatively constant level and high number compared with those of the other two lineages (KK and SK). However, future studies should explore the characteristics of *Wolbachia* strains, that is, how induced phenotypes (reproductive incompatibility, growth and development, and pathogen transmission) respond to the changes in number of *Wolbachia*.

## Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

## Author Contributions

Conceptualization, experiment design, funding acquisition, writing—original draft preparation, writing—review and editing: J.W.; methodology: P.S.; formal analysis: J.W., P.S.; supervision: J.W., J.R. and Y.H.

## Acknowledgments

This study was supported by the National Research Council of Thailand under grant No. 226/2563.

## References Cited

Ali, H., A. Muhammad, S. U. Islam, W. Islam, and Y. Hou. 2018. A novel bacterial symbiont association in the hispid beetle, *Octodonta nipae* (Coleoptera: Chrysomelidae), their dynamics and phylogeny. *Microb. Pathog.* 118: 378–386.

Ausubel, F. M., B. Roger, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 2008. *Current protocols in molecular biology*. John Wiley & Sons, Inc., New York, NY.

Bagheri, Z., A. A. Talebi, S. Asgari, and M. Mehrabadi. 2019. *Wolbachia* induce cytoplasmic incompatibility and affect mate preference in *Habrobracon hebetor* to increase the chance of its transmission to the next generation. *J. Invertebr. Pathol.* 163: 1–7.

Bourtzis, K., S. L. Dobson, Z. Xi, J. L. Rasgon, M. Calvitti, L. A. Moreira, H. C. Bossin, R. Moretti, L. A. Baton, G. L. Hughes, et al. 2014. Harnessing mosquito-*Wolbachia* symbiosis for vector and disease control. *Acta Trop.* 132: S150–S163.

Calvitti, M., F. Marini, A. Desiderio, A. Puggioli, and R. Moretti. 2015. *Wolbachia* density and cytoplasmic incompatibility in *Aedes albopictus*: concerns with using artificial *Wolbachia* infection as a vector suppression tool. *PLoS One.* 10: e0121813.

Chu, C. C., T. A. Gill, M. Hoffmann, and K. S. Pelz-Stelinski. 2016. Inter-population variability of endosymbiont densities in the Asian citrus psyllid (*Diaphorina citri* Kuwayama). *Microb. Ecol.* 71: 999–1007.

Correa, C. C., and J. W. Ballard. 2012. *Wolbachia* gonadal density in female and male *Drosophila* vary with laboratory adaptation and respond differently to physiological and environmental challenges. *J. Invertebr. Pathol.* 111: 197–204.

Dobson, S. L., W. Rattanadechakul, and E. J. Marsland. 2004. Fitness advantage and cytoplasmic incompatibility in *Wolbachia* single- and superinfected *Aedes albopictus*. *Heredity* (Edinb). 93: 135–142.

Dossi, F. C., E. P. da Silva, and F. L. Cónsoli. 2014. Population dynamics and growth rates of endosymbionts during *Diaphorina citri* (Hemiptera, Liviidae) ontogeny. *Microb. Ecol.* 68: 881–889.

Fisher, M. L., J. F. Levine, J. S. Guy, H. Mochizuki, M. Breen, C. Schal, and D. W. Watson. 2019. Lack of influence by endosymbiont *Wolbachia* on virus titer in the common bed bug, *Cimex lectularius*. *Parasit Vectors* 12: 436.

Ge, C., J. Hu, Z. Zhao, A. A. Hoffmann, S. Ma, L. Shen, J. Fang, J. Zhu, W. Yu, and W. Jiang. 2020. Phylogeny and density dynamics of *Wolbachia* infection of the health pest *Paederus fuscipes* Curtis (Coleoptera: Staphylinidae). *Insects.* 11: 625.

Gong, J. T., Y. Li, T. P. Li, Y. Liang, L. Hu, D. Zhang, C. Y. Zhou, C. Yang, X. Zhang, S. S. Zha, et al. 2020. Stable introduction of plant-virus-inhibiting *Wolbachia* into planthoppers for rice protection. *Curr. Biol.* 30: 4837–4845.

Goto, S., H. Anbutsu, and T. Fukatsu. 2006. Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. *Appl. Environ. Microbiol.* 72: 4805–4810.

Guo, Y., A. A. Hoffmann, X. Q. Xu, P. W. Mo, H. J. Huang, J. T. Gong, J. F. Ju, and X. Y. Hong. 2018. Vertical transmission of *Wolbachia* is associated with host vitellogenin in *Laodelphax striatellus*. *Front. Microbiol.* 9: 2016.

Hanboonsong, Y., W. Ritthison, C. Choosai, and P. Sirithorn. 2006. Transmission of sugarcane white leaf phytoplasma by *Yamatotettix flavovittatus*, a new leafhopper vector. *J. Econ. Entomol.* 99: 1531–1537.

Hoffmann, M., M. R. Coy, H. N. Kingdom Gibbard, and K. S. Pelz-Stelinski. 2014. *Wolbachia* infection density in populations of the Asian citrus psyllid (Hemiptera: Liviidae). *Environ. Entomol.* 43: 1215–1222.

Hoffmann, A. A., P. A. Ross, and G. Rašić. 2015. *Wolbachia* strains for disease control: ecological and evolutionary considerations. *Evol. Appl.* 8: 751–768.

Hosseinzadeh, S., M. Shams-Bakhsh, M. Mann, S. Fattah-Hosseini, A. Bagheri, M. Mehrabadi, and M. Heck. 2019. Distribution and variation of bacterial endosymbiont and ‘*Candidatus* Liberibacter asiaticus’ titer in the Huanglongbing insect vector, *Diaphorina citri* Kuwayama. *Microb. Ecol.* 78: 206–222.

Hughes, G. L., R. Koga, P. Xue, T. Fukatsu, and J. L. Rasgon. 2011. *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS Pathog.* 7: e1002043.

Ikeda, T., H. Ishikawa, and T. Sasaki. 2003. Regulation of *Wolbachia* density in the Mediterranean flour moth, *Ephesia kuehniella*, and the almond moth, *Cadra cautella*. *Zool. Sci.* 20: 153–157.

Lopez, V., A. M. Cortesero, and D. Poinot. 2018. Influence of the symbiont *Wolbachia* on life history traits of the cabbage root fly (*Delia radicum*). *J. Invertebr. Pathol.* 158: 24–31.

López-Madrugal, S., and E. H. Duarte. 2019. Titer regulation in arthropod-*Wolbachia* symbioses. *FEMS Microbiol. Lett.* 366: 1–9.

Lu, Y., M. Shiyuan, W. Zhengyan, and W. Sibao. 2019. Prevalence of *Wolbachia* in 10 Tenebrionidae stored-product insects and spatiotemporal infection dynamics in *Tribolium confusum* (Jaquelin Du Val) (Coleoptera: Tenebrionidae). *Grain Oil Sci. Tech.* 2: 85–90.

Mouton, L., H. Henri, M. Bouletreau, and F. Vavre. 2003. Strain-specific regulation of intracellular *Wolbachia* density in multiply infected insects. *Mol. Ecol.* 12: 3459–3465.

Mouton, L., H. Henri, D. Charif, M. Boulétreau, and F. Vavre. 2007. Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. *Biol. Lett.* 3: 210–213.

Noda, H., Y. Koizumi, Q. Zhang, and K. Deng. 2001. Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*. *Insect Biochem. Mol. Biol.* 31: 727–737.

- Ren, S. L., Y. H. Li, D. Ou, Y. J. Guo, J. A. Qureshi, P. A. Stansly, and B. L. Qiu. 2018. Localization and dynamics of *Wolbachia* infection in Asian citrus psyllid *Diaphorina citri*, the insect vector of the causal pathogens of Huanglongbing. *MicrobiologyOpen*. 7: e00561.
- Ruang-areerate, T., P. Kittayapong, E. A. McGraw, V. Baimai, and S. L. O'Neill. 2004. *Wolbachia* replication and host cell division in *Aedes albopictus*. *Curr. Microbiol.* 49: 10–12.
- Serbus, L. R., A. Ferreccio, M. Zhukova, C. L. McMorris, E. Kiseleva, and W. Sullivan. 2011. A feedback loop between *Wolbachia* and the *Drosophila* *gurken* mRNP complex influences *Wolbachia* titer. *J. Cell Sci.* 124: 4299–4308.
- Sumida, Y., M. Katsuk, K. Okada, K. Okayama, and K. Lewis. 2017. *Wolbachia* induces costs to life-history and reproductive traits in the moth, *Ephesia kuehniella*. *J. Stored Prod. Res.* 71: 93–98.
- Thein, M. M., T. Jamjanya, Y. Kobori, and Y. Hanboonsong. 2012. Dispersal of the leafhoppers *Matsumuratettix hiroglyphicus* and *Yamatotettix flavovittatus* (Homoptera: Cicadellidae), vectors of sugarcane white leaf disease. *Appl. Entomol. Zool.* 47: 255–262.
- Tortosa, P., S. Charlat, P. Labbé, J. S. Dehecq, H. Barré, and M. Weill. 2010. *Wolbachia* age-sex-specific density in *Aedes albopictus*: a host evolutionary response to cytoplasmic incompatibility? *PLoS One*. 5: e9700.
- Unckless, R. L., L. M. Boelio, J. K. Herren, and J. Jaenike. 2009. *Wolbachia* as populations within individual insects: causes and consequences of density variation in natural populations. *Proc. Biol. Sci.* 276: 2805–2811.
- Walker, T., P. H. Johnson, L. A. Moreira, L. Iturbe-Ormaetxe, F. D. Frentiu, C. J. McMeniman, Y. S. Leong, Y. Dong, J. Axford, P. Kriesner, et al. 2011. The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature*. 476: U450–U101.
- Wangkeeree, J., P. Tewaruxsa, J. Roddee, and Y. Hanboonsong. 2020a. *Wolbachia* (Rickettsiales: Alphaproteobacteria) infection in the leafhopper vector of sugarcane white leaf disease. *J. Insect Sci.* 20: 20.
- Wangkeeree, J., K. Suwanchaisri, J. Roddee, and Y. Hanboonsong. 2020b. Effect of *Wolbachia* infection states on the life history and reproductive traits of the leafhopper *Yamatotettix flavovittatus* Matsumura. *J. Invertebr. Pathol.* 177: 107490.
- Wangkeeree, J., P. Sanit, J. Roddee, and Y. Hanboonsong. 2021. Phylogeny and strain typing of *Wolbachia* from *Yamatotettix flavovittatus* Matsumura leafhoppers. *Curr. Microbiol.* 78: 1367–1376.
- Werren, J. H., L. Baldo, and M. E. Clark. 2008. *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* 6: 741–751.
- Whelan, J. A., N. B. Russell, and M. A. Whelan. 2003. A method for the absolute quantification of cDNA using real-time PCR. *J. Immunol. Methods.* 278: 261–269.
- Wiwatanaratnabutr, I. 2015. *Wolbachia* infection in leafhoppers and planthoppers: diversity, density and geographic distribution in tropical rice agroecosystems. *J. Asia Pac. Entomol.* 18: 277–282.
- Xi, Z., C. C. Khoo, and S. L. Dobson. 2005. *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. *Science*. 310: 326–328.
- Ye, Y. H., A. M. Carrasco, F. D. Frentiu, S. F. Chenoweth, N. W. Beebe, A. F. van den Hurk, C. P. Simmons, S. L. O'Neill, and E. A. McGraw. 2015. *Wolbachia* reduces the transmission potential of dengue-infected *Aedes aegypti*. *PLoS Negl. Trop. Dis.* 9: e0003894.
- Youichi, K., and Y. Hanboonsong. 2017. Effect of temperature on the development and reproduction of the sugarcane white leaf insect vector, *Matsumuratettix hiroglyphicus* (Matsumura) (Hemiptera: Cicadellidae). *J. Asia Pac. Entomol.* 20: 281–284.
- Zhang, H., K. J. Zhang, and X. Y. Hong. 2010. Population dynamics of noncytoplasmic incompatibility-inducing *Wolbachia* in *Nilaparvata lugens* and its effects on host adult life span and female fitness. *Environ. Entomol.* 39: 1801–1809.
- Zhou, X. F., and Z. X. Li. 2016. Establishment of the cytoplasmic incompatibility-inducing *Wolbachia* strain wMel in an important agricultural pest insect. *Sci. Rep.* 6: 1–9.
- Zhou, W., F. Rousset, and S. O'Neil. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc. Biol. Sci.* 265: 509–515.
- Zug, R., and P. Hammerstein. 2012. Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS One*. 7: e38544.