Establishment of Method for *Wolbachia* Transinfection into the Leafhopper, Vector of Sugarcane White Leaf Disease

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Abstract

Microinjection offers transfection the biological materials that are injected into target insects; it's used to study interaction of insects and injected foreign substances. *Wolbachia* is an intracellular symbiont that can manipulates insect host biology in numerous ways. *Wolbachia* has attracted attention as a potential novel biological control agent against the increase of vector-borne human and insect plant diseases. However, many important insect pests are uninfected or are infected with strains of *Wolbachia* that do not have a critical role and are suitable to be used in the control. The desirable strains of *Wolbachia* can be transferred into new hosts (transinfection), which create infection status in important insect pests. The leafhopper *Matsumuratettix hiroglyphicus* (Matsumura), insect vectors of the phytoplasma that cause white leaf disease in sugarcane and was undetectable of *Wolbachia* infection. The microinjection technique has been no developed in this insect vector; therefore, we have established appropriate procedure microinjection for delivery solution substances and study the successful of *Wolbachia* transinfection into the *M. hiroglyphicus* leafhopper. The results found that PBS solution had no significant difference on survival rate in injection sites between ventral and lateral site of the abdomen (45.83 and 51.85%, respectively) when compared with control solution. In addition, PBS solution also resulted the normal adult longevity (averaged 37.83 days for male and 37.94 days for female) and reproductive traits in the *M. hiroglyphicus* leafhopper. The obtained results was used as procedure microinjection for delivery *Wolbachia* into this insect. After the injection, the injected *M. hiroglyphicus* showed 64.52% positivity (20 out of 31) and 41.67% positivity (5 out of 12) for *Wolbachia* infection at 0 and 7 day post-injection, respectively. The density of *Wolbachia* in injected adult *M. hiroglyphicus* was average as 25.54×10^3 copies of *wsp* gene. This result would provide highlights the importance of further studies on the use of *Wolbachia* as a biological control agent for the leafhopper vector.

***Keywords*:** *Microinjection System, Wolbachia transinfection, Leafhopper, Matsumuratettix hiroglyphicus, Phytoplasma*

1. Introduction

Microinjection system is a process of delivery the foreign substances such as proteins, peptides, RNA interference, drugs and microorganisms into target insects. This system is widely used for study on its genetics, pesticide susceptibility, sterility, interaction and response with injected materials. These are useful for monitoring vector population, alteration of sex ratio, reduced ability to vector human and animal diseases, as well as used as an implication tool for development of control strategy (Chow et al., 2016). For example, the delivery of *Cathepsin-L* dsRNA into nymph stage to study response against *cathepsin-L* gene in the pea aphid (*Acyrthosiphon pisum*) (Sapountzis et al., 2014). The delivery of maize mosaic virus (MMV) into adult stage of corn planthopper vectors (*Peregrinus maidis*) to study infection and transmission efficiency (Yao et al., 2019). In addition, the delivery of three fungi strains into larvae stage to assess fungal toxicity in the mosquito (*Culex quinquefasciatus*) control. The result revealed the mosquito injected with all fungi strains had mortality rates significantly higher than in the control (Bawin et al., 2014) as previous studies demonstrate that microinjection is an interesting and effective technique for foreign substances delivery to study the interaction and response on insects.

Wolbachia is an intracellular symbiont that can manipulate arthropod host biology in numerous ways. Currently, *Wolbachia* has attracted attention as a potential novel biological control agent against the increase of vector-borne human diseases and insect plant diseases (Werren et al., 2008). However, many important insect pests are uninfected or are infected with strains of *Wolbachia* that do not have a critical

role and are suitable to be used in the control. The desirable strains of *Wolbachia* can be transferred into new hosts (transinfection), which create infection status in important insect pests (Hughes and Rasgon, 2014). For example, according to the buffalo flies *Haematobia irritans exigua* transinfected with three strains of *Wolbachia* (*w*AlbB, *w*Mel and *w*MelPop), the result showed a significant decrease in the longevity of female flies of *w*MelPop and *w*Mel-injected (alive less than 50%). Moreover, there was a significant reduction in the total number of eggs laid in all three strains (Madhav et al., 2020). The mosquito *Ae. aegypti* transinfected with three strains of *Wolbachia* (*w*MelCS, *w*Ri and *w*Pip), the result revealed that *w*MelCS showed strong DENV-blocking capacity over 90% (Fraser et al., 2017). The brown planthopper *Nilaparvata lugens* transinfected with *w*Stri strains of *Wolbachia*, *w*Stri induced-CI phenotype show as significantly decrease the egg hatching rates (51.47%), which lower than compatible crosses (80.27-99.77%) (Kawai et al., 2009).

Sugarcane white leaf (SCWL) is a major disease of sugarcane caused by phytoplasma, which is transmitted by the phloem-sucking leafhoppers *Matsumuratettix hiroglyphicus* (Matsumura) and *Yamatotettix flavovittatus* Matsumura. *Wolbachia* detected in natural populations of *Y. flavovittatus* (72.5-100%), *Wolbachia* strain from *Y. flavovittatus* showed strong reproductive incompatibility and completely maternal transmission. However, undetectable of *Wolbachia* infection in *M. hiroglyphicus* (Wangkeeree et al., 2020). This leafhopper is the major vector, because of highly abundance population and greater efficacy of the SCWL phytoplasma transmission. In natural condition, *M. hiroglyphicus* lay their egg in the soil, which 40-50 eggs per one female and revealed more than 70% of hatching. The transmission efficiency of the SCWL phytoplasma was reported at 80-100% (Hanboonsong et al., 2006, Roddee et al., 2018). Currently, there are no resistant sugarcane cultivars or effective means to control SCWL disease. Insect-vector control is one of the crucial factors to limit the spread and transmission of diseases. The *Wolbachia*-based insect control approach has been found to be a long-term, sustaining approach that has no adverse impact on natural ecosystems.

The microinjection system has been no developed in the *M. hiroglyphicus* leafhopper, therefore, the aim of the present study was to establish this system for delivery solution substances. We examined the effects of microinjection system on survival rate, adult longevity and fecundity. In addition, the obtained appropriate microinjection system was used for delivery *Wolbachia* into this insect. The results of the study will lead to the development for future on the leafhopper vector control strategies.

2. Objectives

1) To find out the appropriate procedure microinjection technique for the *M. hiroglyphicus* leafhopper

2) To study the efficiency of *Wolbachia* transinfection into the *M. hiroglyphicus* leafhopper.

3. Materials and Methods

3.1 Leafhopper populations

Adult *Y. flavovittatus* and *M. hiroglyphicus* were collected from sugarcane fields using light traps. The specimens were maintained in the sugarcane plant-cages and transferred to the laboratory. The adult leafhoppers were reared on sugarcane plants in a controlled laboratory until the next generation emerged. After one or two generations, the leafhoppers from the stock were used for the experiments. DNA extraction was also performed from some of natural populations to confirm *Wolbachia* infection.

3.2 DNA extraction and *Wolbachia* detection

The insects collected from fields were extracted their genomic DNA using the phenol-chloroform method (Ausubel et al., 2008) and the extraction protocol for leafhoppers described by Wangkeeree et al. (2020). In brief, each individual leafhopper was ground in a DNA extraction buffer (200 mM Tris pH 8.0, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.1 mg/mL proteinase K; Invitrogen, Carlsbad, CA, USA) and incubated at 37 ◦C for 24 h. The DNA was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), followed by 5 min of centrifugation at 10,000 × *g* at 4 ◦C.

The supernatant was transferred to another vial, and an equal volume ratio of chloroform:isoamyl alcohol (24:1) was added. After 5 mins of centrifugation at 10,000 × *g* at 4 ◦C, the DNA was precipitated with 3 M sodium acetate and isopropanol. The resulting pellet was washed with 70% absolute ethanol, air dried, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA), before being stored at -20 ◦C until further use.

The leafhoppers were tested for the presence of *Wolbachia* by PCR using *Wolbachia*-specific primers. The 610-bp *wsp* surface protein marker was amplified using the forward primer 81F (5′-TGGTCCAA-TAAGTGATGAAGAAAC-3′) and the reverse primer 961R (5′-AAAAAT-TAAACGCTACTCCA-3′) (Zhou et al., 1998). PCR amplifications were performed in a final reaction volume of 20 µL containing 2 µL of template DNA, 1 × reaction buffer, 2.5 mM $MgCl_2$, 0.5 µM primers, 0.2 mM dNTPs, and 1 U *Taq* DNA polymerase (Invitrogen).

PCR consisted of the following steps: an initial denaturation (94 ◦C, 5 min), followed by 30 cycles of denaturation at 95 ◦C (1 min), annealing at 55 ◦C (1 min), and extension at 72 ◦C (1 min), and a final extension at 72 ◦C (10 min). The amplicons were analyzed by gel electrophoresis and visualized under ultraviolet illumination.

3.3 Development of a microinjection system for the leafhopper *M. hiroglyphicus*

To optimize this technique, we performed experiments injecting the insects with two parameters including the injection site on the leafhopper body and the buffer solution. Adult *M. hiroglyphicus* were injected using injector apparatus Nanoject II (Drummond Scientific, Broomall, PA, USA) set to slow speed, and with an injection volume 46 nl to ensure a low mortality rate in the injected leafhopper.

Before injection, the leafhoppers were placed on ice and immobilized by cold for 2 mins. Two different injections sites including ventral and lateral region of the abdomen were examined (Figure 1). For each region, two types of buffer solutions including Phosphate-Buffered Saline (PBS) (130 mM NaCl, 7 mM $Na_2HPO_4 \cdot 2H_2O$, 3 mM $NaHPO_4 \cdot 2H_2O$, pH 7.0) and Ringer's buffer (182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.2) were injected. Leafhoppers that were injected with deionized water were used as control solution. Therefore, our experiment was designed as completely randomized design, which include 6 treatments are as follows: 1) ventral abdomen with deionized water, 2) ventral abdomen with PBS, 3) ventral abdomen with Ringer, 4) lateral abdomen with deionized water, 5) lateral abdomen with PBS and 6) lateral abdomen with Ringer. Number of leafhoppers were served as number of replications in each treatment (one leafhopper as one replication).

After microinjection, the survival rates were recorded after 72 h., and compared with the leafhoppers without injection. Surviving female *M. hiroglyphicus* were released into cages with sugarcane leaves (one male and one female per cage) for mating. Longevity was recorded for each adult. After 10-14 days, the females laid eggs in the soil, number of eggs and hatched eggs were recorded daily. The fresh hatching nymphs were transferred to the new plant cages and maintain until adult emergence, then each individual was sexed (Figure 2). The parent and offspring were collected for DNA extraction and detection for *Wolbachia* prevalence.

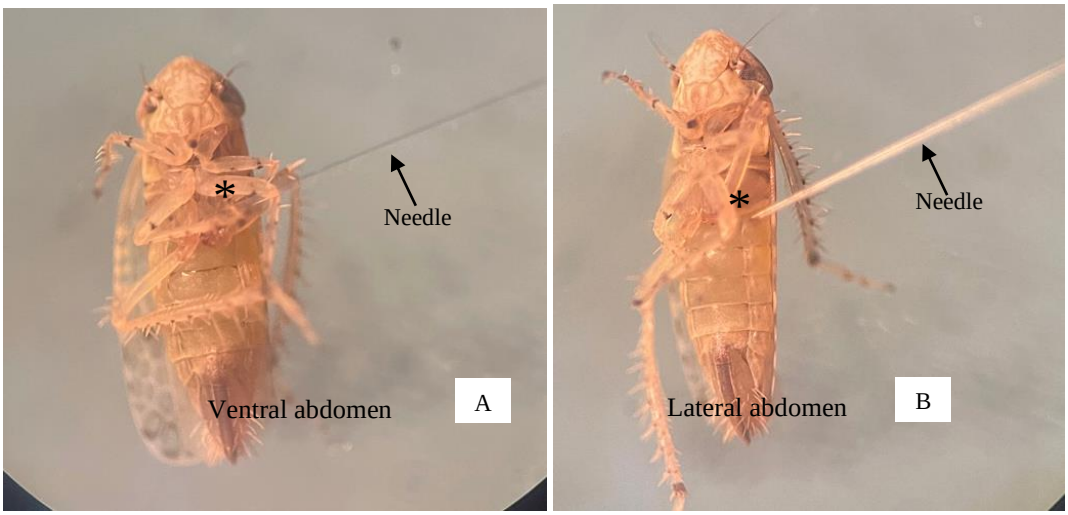

Figure 1 Injection sites: A) ventral and B) lateral region of the abdomen

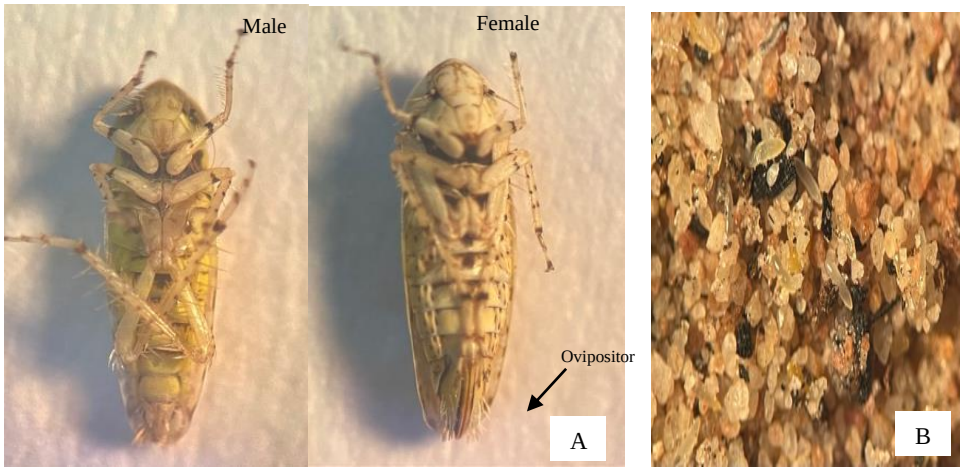

Figure 2 A) Male and female of adult *M. hiroglyphicus* and B) Characteristics of deposited eggs of *M. hiroglyphicus*

3.4 *Wolbachia* transinfection

3.4.1 Preparation of *Wolbachia* source

For injection experiments, eggs from *Wolbachia*-infected *Y. flavovittatus* were used as a donor insect. The *Wolbachia*-uninfected *M. hiroglyphicus* was used as the recipient line for transinfection. *Wolbachia* strains were directly isolated from eggs of *Wolbachia*-infected *Y. flavovittatus* and introduced into adult stages of *M. hiroglyphicus*. In brief, *Wolbachia*-infected eggs (10-15 eggs) were transferred to 1.5-ml tube with 2 µL fresh buffer and homogenized (~10 strokes at room temperature with the pellet pestle), The homogenate was then transferred into a clean tube placed on ice until used for injection (<5 h).

3.4.2 *Wolbachia* infection dynamics in injected *M. hiroglyphicus*

Wolbachia infection rates in injected *M. hiroglyphicus* were examined using *wsp* primers (81F/691R) were used for *Wolbachia* detection as previously described. The density of *Wolbachia* in the whole body of injected *M. hiroglyphicus* was measured using quantitative PCR (q-PCR) based on the *wsp* gene. Procedures, including SYBR Green™ Realtime PCR Master Mix, PCR primers, standard DNA, and PCR conditions. Males and females were sampled at 0 and 7 day after injection. The q-PCR results were translated into the number of *Wolbachia* in the whole body of *M. hiroglyphicus.*

3.5 Statistical analysis

The normality of the data obtained (except on survival rate) were first checked using the Kolmogorov-Smirnov test. To normalize the data, log transformation was used for adult longevity, and number of eggs laid per female, whereas arcsine square root transformation was used for hatchability and female in offspring. Survival rate was calculated and tested for statistical significance (alive = 1, death = 0). Data were analyzed using one-way analysis of variance (ANOVA), and means were compared using the Tukey HSD test. All statistical analyses were carried out using IBM SPSS Statistics 20.

4. Results and Discussion

4.1 Detection of *Wolbachia* in natural populations of leafhopper

Wolbachia detection was examined in natural populations of the leafhopper *Y. flavovittatus* and *M. hiroglyphicus*. *Wolbachia* infection was 100% in *Y. flavovittatus*, whereas no infection rate was found in *M. hiroglyphicus* (Table1).

Table 1 Detection of *Wolbachia* in natural populations of the leafhopper

Leafhopper populations	No. of tested (No. of positive)		% Positive both sexes
	Male	Female	
Y. flavovittatus	20 (20)	20 (20)	100%
M. hiroglyphicus	20 (0)	20 (0)	0%

4.2 Effect of microinjection technique on biology traits of *M. hiroglyphicus*

The survival rate of treated-adult *M. hiroglyphicus* after injection is shown in Table 2. The leafhoppers remain 50.00% and 44.74% after injected with control solution (deionized water) at ventral and lateral region of the abdomen respectively. This was no significant difference when injected with PBS solution, which revealed 45.83 -51.85% of survival rate from both injection sites. For Hemipteran insect, the intersegment at ventral and lateral region of the thorax and abdomen are the most suitable for inserting the capillary needle due to the fact that these positions are the flexible and thin tissues (Xu et al., 2015). However, a size of capillary needle is critical factor for survival rate. The capillary needle used should be sharp and fine-tip to allow easy penetration through the membrane of insect (Li et al., 2017). In this experiment, the procedure using capillary needle for injection may be a side effect on survival rate of the *M. hiroglyphicus* leafhoppers, resulting in half of mortality when compared with the leafhopper without injection.

However, the Ringer solution had lowest survival rates, which 25.00% and 21.74% for ventral and lateral region of the abdomen, respectively. These showed significantly difference ($P < 0.01$) from PBS buffer and control solution. In generally, Ringer solution is safe but may cause swelling and fluid buildup in tissue if overused. In addition, Tris-HCl is a component of Ringer solution that can exert toxic effect on nervous system (Gillespie and McKnight, 1976). Nevertheless, the appropriate solutions that deliver into insects depend on types of solution and insect species. PBS and Ringer solutions have no effect on survival rate when injected into the fly *Drosophila simulans*, which averaged 51.2% and 66.7% respectively (Xi and Dobson, 2005).

Table 2 Survival rate (%) of injected adult *M. hiroglyphicus* at 72 h after injection

Injection sites	Injected solution	No. of injected / No. of survival (%)		Survival rate of both sexes (%) ± SE
		male	female	
Control (without injection)		15/15 (100%)	10/10 (100%)	100.00 ± 0.00 a[1/]
Ventral abdomen	deionized water	15/8 (53.33%)	13/6 (46.15%)	50.00 ± 3.59 b
	PBS	8/4 (50%)	16/7 (43.75%)	45.83 ± 3.13 b
	Ringer	10/2 (20%)	10/3 (30%)	25.00 ± 5.00 c
Lateral abdomen	deionized water	13/6 (46.15%)	25/11 (44%)	44.74 ± 1.08 b

Injection sites	Injected solution	No. of injected / No. of survival (%)		Survival rate of both sexes (%) ± SE
		male	female	
	PBS	12/6 (50%)	15/8 (53.33%)	51.85 ± 1.67 b
	Ringer	13/2 (15.38%)	10/3 (30%)	21.74 ± 7.31 c
F				43.284
P[2/]				0.000

[1/] Mean (±SE) differ significantly at P < 0.01 (**) (ANOVA)
[2/] Values in a column followed by different letters are significantly different at P < 0.05 (Tukey HSD test)

Adult longevity of injected adult *M. hiroglyphicus* is summarized in Table 3. No significant difference among treatment was detected in adult longevity, which averaged 37.83 days for male and 37.94 days for female. This result is similar to a previous study for the adult *M. hiroglyphicus* in which longevity of natural populations were 30-42 days (Hanboonsong et al., 2002).

Table 3 Adult longevity (in days, mean ± SE) of injected adult *M. hiroglyphicus*

Injection sites	Injected solution	Adult longevity in days, mean ± SE (Total)[1/]	
		male	female
Ventral abdomen	deionized water	36.36 ± 1.23 (11)	39.82 ± 1.88 (11)
	PBS	39.25 ± 2.39 (4)	38.00 ± 2.48 (7)
	Ringer	36.50 ± 2.50 (2)	37.33 ± 1.20 (3)
Lateral abdomen	deionized water	37.67 ± 1.86 (6)	38.27 ± 1.48 (11)
	PBS	38.67 ± 1.54 (6)	35.88 ± 2.95 (8)
	Ringer	38.50 ± 2.50 (2)	38.33 ± 0.88 (3)
F		0.444	0.341
P[2/]		0.814	0.885

[1/] Values in parentheses are the number of individuals tested.
[2/] NS, Not significant at the 5% level (ANOVA)

To determine the effect of microinjection technique on copulation success, number of eggs laid, hatchability, and females in offspring of injected female *M. hiroglyphicus* were recorded (Table 4). All treatments (36 pairs) had a copulation success of 100%. No significant difference among treatment was found in number of eggs laid (mean = 38.09 eggs/female), hatchability (approximately 84.37%), and number of females in offspring (approximately 50.69%). Our results are in agreement with previous studies, in natural population of female *M. hiroglyphicus* were 20.7-48.6 eggs laid/female, over 80% of hatchability and approximately 52.98% in females in offspring (Kobori and Hanboonsong, 2017). However, a number of pairs seem to be low due to the survival females that remains after injection were investigated. Though, number of specimens should be increase as two or three-fold for further injection.

Table 4 Copulation success, number of eggs laid, hatchability, and females in offspring of injected female *M. hiroglyphicus*

Injection sites	Injected solution	No. of pairs	Copulation success (%)	Eggs laid/ female (means ± SE)	Hatchability (% ± SE)	Females in offspring (%)
Ventral	deionized water	7	100	40.86 ± 3.17	85.38 ± 2.71	50.10 ± 1.74 ab[1/]
abdomen	PBS	7	100	39.43 ± 4.75	82.24 ± 1.39	53.94 ± 4.45 ab

Injection sites	Injected solution	No. of pairs	Copulation success (%)	Eggs laid/ female (means ± SE)	Hatchability (% ± SE)	Females in offspring (%)
	Ringer	3	100	41.00 ± 4.04	85.06 ± 2.90	56.35 ± 4.83 a
Lateral abdomen	deionized water	8	100	36.63 ± 4.20	84.15 ± 2.80	51.55 ± 2.47 ab
	PBS	8	100	32.63 ± 5.44	79.60 ± 6.77	49.16 ± 2.05 ab
	Ringer	3	100	38.00 ± 3.51	89.76 ± 1.73	43.05 ± 2.01 b
F				0.485	0.515	1.280
P[2/]				0.785	0.763	0.300

[1/] Values in a column followed by different letters are significantly different at P < 0.05 (Tukey HSD test)
[2/] NS, Not significant at the 5% level (ANOVA)

4.3 *Wolbachia* infection dynamics in injected *M. hiroglyphicus*

The presence of *Wolbachia* in injected *M. hiroglyphicus* were examined 0 and 7 days post-injection (Table 5). The infection rate of *Wolbachia* was 64.52% at 0 day post-injection. This was significant difference when compared at 7 day post-injection, which was 41.67%. *Wolbachia* infection was decreases along with increasing time.

However, no significant difference in the infection density of *Wolbachia* in the whole body of the injected *M. hiroglyphicus* at 0 and 7 day post-injection ranged from 18.70 - 20.86 x 10^3 and 33.89 - 28.72 x 10^3 *wsp* copies, respectively (Figure 3). On the contrary, a previous study of the *Wolbachia* density in injected flies (*Haematobia irritans exigua*) showed an initial significantly decreasing to approximately day 7 and increase in *Wolbachia* titre to day 11 (Madhav et al., 2020).

Table 5 *Wolbachia* infection after *Wolbachia* transinfection of injected *M. hiroglyphicus*

Day post-injection	No. of injected / No. of positive (%)		Positive both sexes (% ± SE)
	Male	Female	
0	17/11	14/9	64.52 ± 0.21[1/]
7	7/3	5/2	41.67 ± 1.43
F			254.775
P			0.034

[1/] Mean (±SE) differ significantly at P < 0.05 (*)

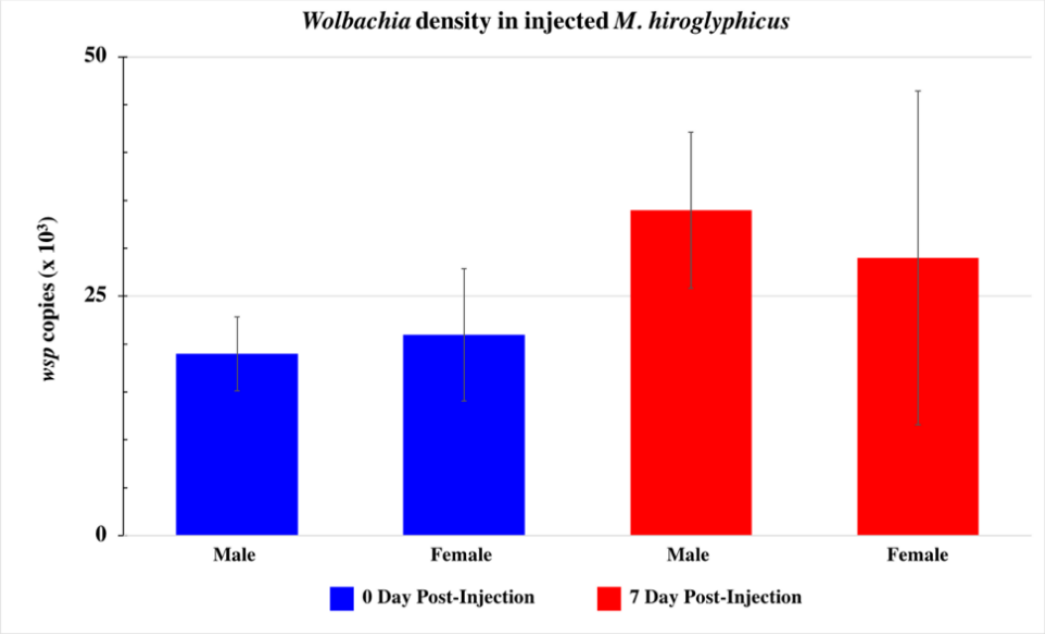

Figure 3 *Wolbachia* density in injected *M. hiroglyphicus* at 0 and 7 day post-injection. Values represent the mean (± SE) of *wsp* gene copies per 50 ng of host genomic DNA. No significant difference determined using Tukey's HSD test ($F = 0.560$, $P > 0.05$)

5. Conclusion

In this study, we find out the appropriate procedure of microinjection technique for delivery *Wolbachia* into the *M. hiroglyphicus* leafhopper. Our results revealed that PBS is suitable mixed solution with *Wolbachia* for injection. Moreover, *Wolbachia* were detected in the injected leafhoppers. This finding is the first step forward for further research, for example, the investigation of the stability and distribution of *Wolbachia* after injection and effect of *Wolbachia* on the host's biology traits. These include *Wolbachia*-induced incompatibility and interfere disease transmission.

6. Acknowledgements

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