



## Research article

Relationship between *Wolbachia* infection in *Culex quinquefasciatus* and its resistance to insecticideAtieh Shemshadian<sup>a</sup>, Hassan Vatandoost<sup>a,b,\*</sup>, Mohammad Ali Oshaghi<sup>a</sup>,  
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## ABSTRACT

Many studies have been done on the various factors affecting resistance to insecticides. The relationship between *Wolbachia* bacteria and resistance to insecticides is one of the factors that has attracted a lot of attentions. *Wolbachia* are obligatory intracellular endosymbionts that naturally occur in a wide range of arthropods and nematodes, including the mosquito *Culex quinquefasciatus*.

Initially, the presence of bacteria was proved by molecular assays. Then the resistance level of *this* species was evaluated in adults against DDT 4.0% and deltamethrin 0.05% using the standard WHO guideline. After elimination of *Wolbachia* by tetracycline and its proof by molecular assays, the susceptibility tests were conducted again on uninfected line. Finally, the two lines were compared in terms of responding to insecticides. The findings indicated that there is no significant correlation between susceptibility of two lines in response to DDT 4.0% while they represented a significant correlation for deltamethrin ( $P = 0.00$ ).

We propose that *Wolbachia* bacteria increase the susceptibility to deltamethrin but they show neutral effect on DDT susceptibility in *Cx. quinquefasciatus*. However, more studies on other vectors and insecticides still need to be done.

## 1. Introduction

Resistance to insecticides has become an important public health hazard and has increased in all the medically important insects (Rivero et al., 2010). This issue has caused a major problem to the control of mosquito vectors, specially *Cx. quinquefasciatus* which is born in wastewaters and is exposed to different kinds of insecticides (Liu et al., 2013). It is expected that insecticide resistance will directly contribute to the reemergence of vector-borne diseases (Brogdon and McAllister, 1998). Due to these issues, interests in biological control methods have been raised. One of the alternative method is using *Wolbachia* bacteria. The widespread bacteria *Wolbachia* have been detected in all classes of arthropods, as well as in the nematode family (Zug and Hammerstein, 2012). These bacteria are intracellular, which are inherited maternally (Zhu et al., 2012). *Wolbachia* can influence their hosts by reproductive manipulations including cytoplasmic incompatibility, feminization, male killing, and parthenogenesis (Wang et al., 2016; Turley et al., 2009; Werren, 1997). Furthermore, *Wolbachia* affect other parameters in their

host like fitness, blood feeding, pathogen interference, disease transmission, immune system, probing behavior, host temperature preference, and insecticide resistance (Hague et al., 2020; Caragata et al., 2016; De Almeida et al., 2011; Glaser and Meola, 2010; Turley et al., 2009; Kambris et al., 2009; Moreira et al., 2009; Duron et al., 2006; Berticat et al., 2002). Several studies have been carried out to investigate the relationship between *Wolbachia* infection and insecticide resistance. The results of some of these studies illustrate that *Wolbachia* increase the host susceptibility to insecticides, while others show the opposite outcomes. Berticat et al. (2002) showed that the *Wolbachia* density was strongly influenced by the presence of resistance genes in *Culex pipiens*. Their results revealed that the resistant mosquitoes show higher levels of *Wolbachia* infection than susceptible species, despite having the same genetic background. Their major assumption was the disability of mosquito which controls the *Wolbachia* density due to presence of resistance genes. Similar results were shown in Duron et al. (2006). They compared the *Cx. pipiens* strains which shared a common genetic background but differed from the resistance alleles and *Wolbachia* loading. Subsequently,

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Kontsedalov et al. (2008) indicated that the presence of specific symbionts can be associated with resistance to insecticides in *Bemisia tabaci*. They reported that, more susceptibility was observed to some insecticides in *Rickettsia* positive population. In the same study, Ghanim and Kontsedalov (2009) reported the correlation between the bacterial densities and insecticide resistance level in *Bemisia tabaci*. Their result suggested that the more symbionts a pest population gains, the more susceptible to insecticides they will be. The studies about interaction between symbiont presence and resistance to insecticide continued by Echaubard et al. (2010). They studied the evolution of the *Wolbachia* densities in laboratory and field populations of *Cx. pipiens* over the course of 50 generations. Their results indicated that in the old population the *Wolbachia* densities were higher in insecticide-resistant individuals than insecticide-susceptible individuals for both sexes.

Following these studies, concerns about the negative effects of *Wolbachia* on vector control were raised. In a study by Endersby and Hoffmann in 2012, the resistance levels in *Aedes aegypti* *Wolbachia* infected and uninfected lines to bifenthrin, *Bacillus thuringiensis*, temephos and s-methoprene were evaluated. Results showed that there was no adverse effect of *Wolbachia* infection on chemical control of *Ae. aegypti*. However, the recent studies have revealed contradictory results. Li et al. (2018) suggested that *Serratia* and *Wolbachia* infection might increase the resistance to buprofezin in *Laodelphax striatellus*. In another study, Li et al. (2020) indicated that *Wolbachia* are involved in insecticide resistance in some genetic background of *Laodelphax striatellus* while having no effect in others. To help with having a clearer understanding towards the influence of *Wolbachia* bacteria on insecticide resistance, we studied the levels of resistance in two *Wolbachia* infected and uninfected lines of *Cx. quinquefasciatus* to DDT and deltamethrin. The result of this study may offer a perspective on the possible role of *Wolbachia* in insecticide resistance.

## 2. Materials and methods

### 2.1. Study area

A residential coastal area located on the edge of Bandar-Abbas, Hormozgan province near the Persian Gulf, was selected for mosquito collection (Suru, 27°10N-56°15E). Suru was a district location on the edge of the city (see Figure 1).



**Figure 1.** Sampling location in Suru, Bander-Abbas Port, Hormozgan province, southern Iran.

### 2.2. Mosquito collection and rearing

The samples were collected in stagnant contaminated water from both abandoned boats and holes. The larvae and pupae along with the water and plants of the breeding places, were transported to the insectary at Bandar-Abbas Training and Research Station. All mosquito larvae and adults were reared in an insectary at the temperature of  $30 \pm 5$  °C,  $70 \pm 5\%$  Relative Humidity and photoperiod of 12:12 [L: D].

Larvae and adults were kept in 42 cm by 25 cm by 25 cm trays and 65 cm<sup>3</sup> cages, respectively. Larvae were fed with fish flake and adults with sucrose solution and blood feeding on birds.

### 2.3. Detection of *Wolbachia*

DNA extraction was performed using Collins DNA extraction method (Collins et al., 1987) from the whole adult male and female bodies. In this study, *Wolbachia* surface protein gene (*wsp*) was detected based on the standard protocol (Braig et al., 1998; Zhou et al., 1998) to prove the presence of these bacteria using semi-nested PCR. At the beginning of the study, general *wsp* primers as formerly depicted in Braig et al., (1998), were used.

General primers, *wsp* 81F: 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and *wsp* 691R: 5'-AAAAATTAACGCTACTCCA-3', which are able to amplify a DNA fragment in the range of 590–632 bp depending on the individual *Wolbachia* strain, were utilized. The first PCR product was used as a template for the second PCR. In second PCR, the of 501 bp fragment was amplified using a second set of primers, 183F:5'-AAGGAACCGAAGTTCATG-3' and 691R: 5'-AAAAATTAACGCTACTCCA-3'. The amplifications were checked by agarose electrophoresis. *wsp* gene sequencing was performed by primers 183F and 691R, and the results were sent in the shape of Chromos curves. The similarity between the obtained sequences verified the effectiveness of using Clustal W2 software. The NCBI BLAST server was used to compare with GenBank sequences records.

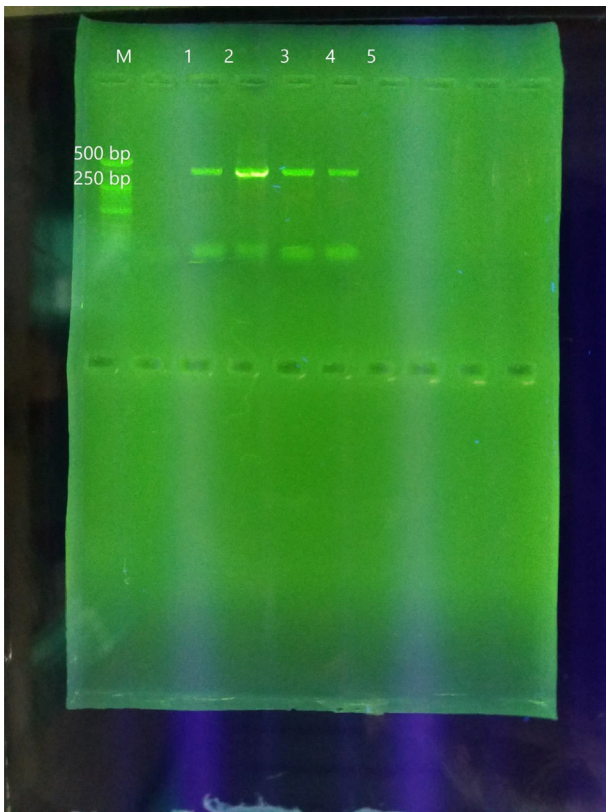
### 2.4. Insecticide susceptibility test (before treatment)

The susceptibility tests were done using test papers treated with DDT 4.0% and deltamethrin 0.05% supplied by the Vector Control Research Unit, School of Biological Sciences, 11800 Minden, Penang, Malaysia.

Adult susceptibility tests were performed according to WHO standard protocol (World Health Organization, WHO 2016). Twenty-five freshly emerged sugar fed females, 2–3 days old, were exposed to DDT 4.0% and deltamethrin 0.05% impregnated paper on the WHO standard test kit. Tests with insecticide free papers were conducted in parallel and served as the control. Ten replicates were conducted for each insecticide, and two replicates were conducted for the control. Once the exposure time ended, both mosquito groups were allowed to recover in holding tubes supplied with 10% sucrose solution. Mortality was verified after a 24-h insecticide exposure. The susceptibility tests were conducted separately on three different generations of *Cx. quinquefasciatus*, the field population, F3, and F6.

### 2.5. Tetracycline treatment

Three different protocols were used to remove *Wolbachia* from *Cx. quinquefasciatus* including treatment of eggs, adults, and larvae. The former two attempts failed due to high mortality but the larvae treatment showed desirable results. Tetracycline hydrochloride (Sigma-Aldrich) at the concentration of 0.05 mg/ml was used <https://www.powerthesaurus.org/applied/synonyms> for the first larval stage treatment and was applied over three generations. The experiments were planned according to the previous studies (Yen and Barr, 1973; Rasgon and Scott, 2003). Tetracycline solution was buffered to pH 7 using 1 M solution of unbuffered Tris (pH 11). To avoid larval mortality, addition of the food at the beginning of rearing was essential. Additionally, the treated larvae were kept in the dark to prevent the oxidation of the antibiotic. Before



**Figure 2.** First Semi-Nested PCR products (~630) of *Wolbachia wsp* gene of *Cx. quinquefasciatus* specimens. Lanes: M, 50bp molecular weight marker, 1: negative control, 2: positive control, 3–5: Bandar Abbas population of *Cx. quinquefasciatus*.

the colony was used for the susceptibility tests, mosquitoes were reared for three generations under the standard condition (without antibiotics) to avoid any antibiotic side effects. *Wolbachia* PCR assay was done on some specimens of each generation after treatment.

**2.6. Insecticide susceptibility test (after treatment)**

The second stage susceptibility tests were performed using mosquitoes of the first, second, and third generations of the uninfected line according to the WHO standard protocol.

**2.7. Statistics analysis**

Mortality rates of *Cx. quinquefasciatus* after 24 h of recovery were calculated for each insecticide and strain. Then, they were corrected

using Abbott's formula when the mortality rate in the control group is higher than 5%. The following criteria were used for interpretation of the resistance status of *Cx. quinquefasciatus* according to the WHO criteria. The resistance, tolerance, and susceptibility were ranked for groups where the mortality rates were less than 90 percent, between 90 and 98 percent, and higher than 98 percent respectively. Univariate analysis of variance (ANOVA) was conducted after an arcsin transformation of the mortality rate to determine differences in mosquito mortality rates among strains. The significant differences of the mean of mortality rates were compared using either Tukey or Games-Howell tests. the standard error (SE) was calculated using the following formula:  $SE = \sqrt{p \times q/n}$  where *p* is the mortality rate, *q* is 1 minus the mortality rate, and *n* is the sample size (Figures 4, 5, and 7, 8, 9, and 10).

**3. Results**

**3.1. Wolbachia prevalence in wild population**

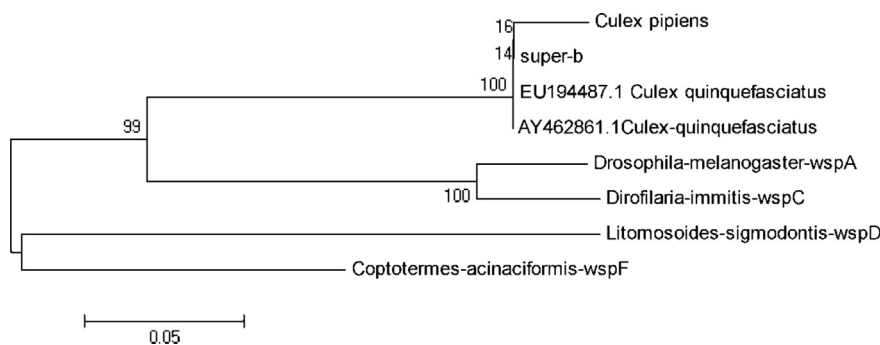
Results of the current study showed the presence of *Wolbachia* bacteria in all males and females of each generations, selected from the Bandar Abbas population. The infection was detected by Semi-Nested-PCR assay using *wsp* gene. The amplicons of first and second runs of Semi-Nested-PCR assay were ~600 and 500 bp respectively (Figure 2).

Semi-Nested-PCR product of the *Wolbachia wsp* gene, detected in Bandar Abbas population of *Cx. quinquefasciatus*, was sequenced and then submitted to Genbank (Accession Number: MK360157). The BLAST results of the sequences demonstrated 99% similarity to *Wolbachia* endosymbiont in *Cx. pipiens* with the Accession Number KT964228.1 which belongs to Turkey. All the wild specimens were found to shelter the *Wolbachia* strain belonging to the Pip group of B supergroup (wPipB).

The *Wolbachia* strain, identified in this study, was subjected to a molecular phylogenetic analysis with available sequence data of 30 other *Wolbachia* strains from the Genbank database belonging to orders Diptera, Hymenoptera, Lepidoptera, Odonata, Hemiptera, and Arachnida. The results showed 99% similarity between *Wolbachia* bacteria in *Cx. quinquefasciatus* of the Bandar Abbas population, and *wsp* gene sequence in *Cx. quinquefasciatus*, and *Cx. pipiens* from the United Kingdom, China, India, Taiwan, Iran, and Turkey. A phylogenetic tree was made using the neighbor-joining method of MEGA7 software, based on the 500 bp of *wsp* sequences. The specimens were compared to other *Wolbachia* strain sequences in the Genbank. These sequences belong to hosts such as mosquitoes (*Culex*), flies (*Drosophila*), nematodes (*Litomosoides*, *Dirofilaria*) and termites (*Coptotermes*) (Figure 2) (see Figure 3).

**3.2. Insecticide susceptibility test (before treatment)**

In this stage of the research, three generations of *Cx. quinquefasciatus* were tested. The field population, the third generation, and the sixth generation were coded as Field strain-W+, Field strain-F3-W+ and Field



**Figure 3.** The phylogenetic tree derived from 554 bp of *wsp* sequences of *Wolbachia pipiens* hosts using the neighbor-joining method. The numbers on the branches are the bootstrap values. Scale below the tree is the amount of genetic differences between species.

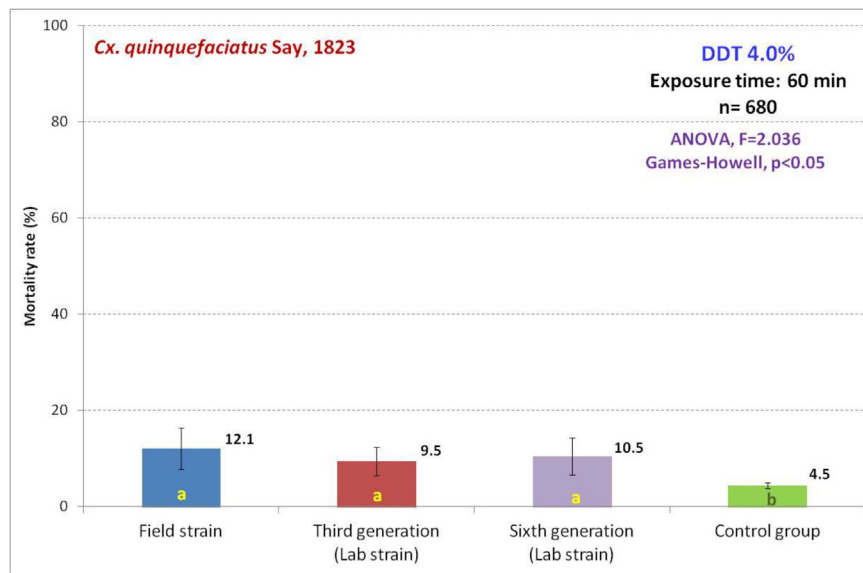


Figure 4. Comparison of susceptibility levels of different *Cx. quinquefasciatus* populations to DDT 4.0%.

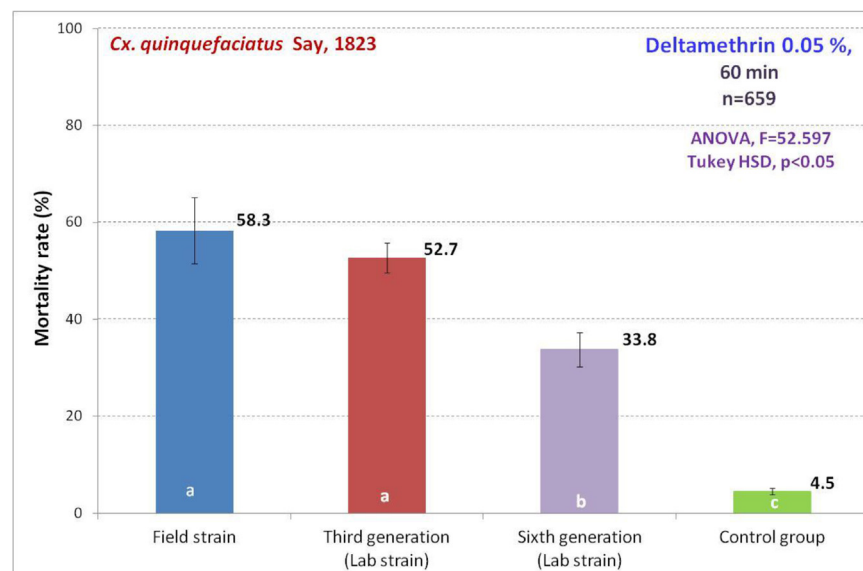


Figure 5. Comparison of susceptibility levels of different *Cx. quinquefasciatus* populations to deltamethrin 0.05%.

strain-F6-W+ respectively. The mortality rate of female *Cx. quinquefasciatus*, exposed to the DDT of 4.0%, was 12.1% in the field strain, 9.5% in the third generation (F3) and 10.5% in the sixth generations (F6) (Figure 4). Mortality rate comparison between different isolates indicated stability in the resistance level to DDT in the population, in spite of breeding six generations in insectary. For the deltamethrin of 0.05%, the rates in the field strain, F3, and F6 were 58.3, 52.7, and 33.8%, respectively (Figure 5). The results revealed that resistance increased the generations progressed. This data showed that the *Cx. quinquefasciatus* population was highly resistant to both studied insecticides (Table 1).

### 3.3. *Wolbachia* detection in treated population

To ensure bacteria were cleared after treating population with tetracycline, *Wolbachia* PCR assay was conducted using the same protocol and primers which were mentioned earlier (Braig et al., 1998; Zhou et al., 1998). According to our results, no bacteria were detected (Figure 6).

### 3.4. Insecticide susceptibility test (after treatment)

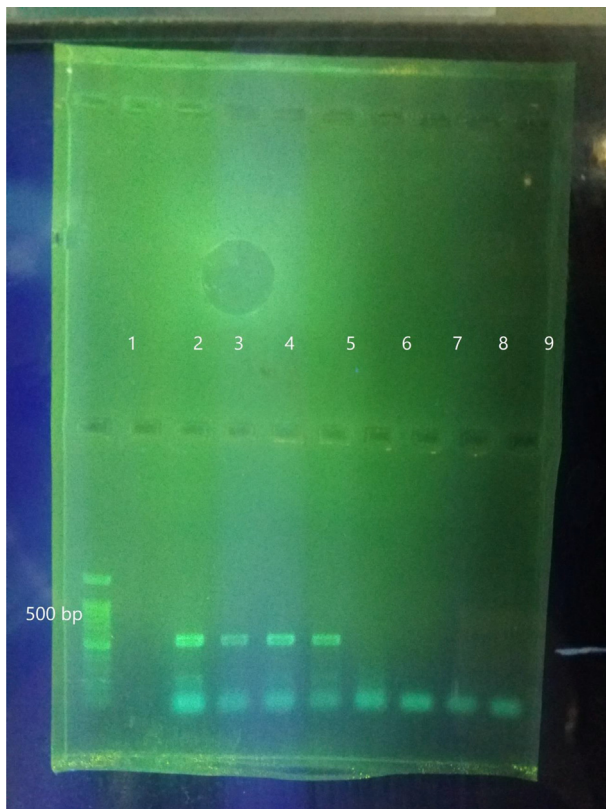
After tetracycline treatment and bacteria clearing verification, the population was reared for three more generations under the standard condition to restore the normal bacterial flora and avoid antibiotic pressure. These generations were coded as F1 Lab W-, F2 Lab W-, and F3 Lab W-. The mortality rates of female *Cx. quinquefasciatus* exposed to DDT = 4.0% were  $10.5 \pm 3.9$ ,  $8.9 \pm 2$ , and  $6.5 \pm 1.7$  in F1 Lab W-, F2 Lab W-, and F3 Lab W-, respectively (Figure 7). Despite the elimination of bacteria, the results indicated no change in resistance level of *Cx. quinquefasciatus* against DDT. The mortality rate of female *Cx. quinquefasciatus*, exposed to deltamethrin, changed to  $33.8 \pm 3.5$  in F1 Lab W-,  $32.7 \pm 6$  in F2 Lab W- and,  $38.8 \pm 4.4$  F3 in Lab W- (Figure 8). There was no alteration in the resistance level of the *Wolbachia* free population to deltamethrin (Table 2).

### 3.5. Comparison of mortality among treated and untreated populations

After conducting the susceptibility test on treated (F1 Lab W-, F2 Lab W-, and F3 Lab W-) and untreated (Field strain-W+, Field strain-F3-W+,

**Table 1.** Susceptibility levels to DDT 4.0% and deltamethrin 0.05% in three generations of *Wolbachia* positive *Cx. quinquefasciatus*.

Generation	Insecticide	No dead	No tested	Mortality $\pm$ SE	Corrected Mortality
Field strain-W+	DDT 4.0%	23	96	7.3 $\pm$ 24	7.3 $\pm$ 12.1
	deltamethrin 0.05%	62	97	6.8 $\pm$ 63.9	6.8 $\pm$ 58.3
	Control	10	74	3.4 $\pm$ 13.5	-
Field strain-F3-W+	DDT 4.0%	23	243	3 $\pm$ 9.5	-
	deltamethrin 0.05%	127	241	3.1 $\pm$ 52.7	-
	Control	0	50	0	-
Field strain-F6-W+	DDT 4.0%	16	153	3.9 $\pm$ 10.5	-
	deltamethrin 0.05%	45	133	3.5 $\pm$ 33.8	-
	Control	0	64	0	-

**Figure 6.** Second Semi-Nested PCR products (~500) of *Wolbachia wsp* gene of *Cx. quinquefasciatus* specimens. Lanes: M, 50bp molecular weight marker, 1: negative control, 2: positive control, 3–5: Bandar Abbas population of *Cx. quinquefasciatus*, 6–9: treated Bandar Abbas population of *Cx. quinquefasciatus*.

and Field strain-F6-W+) populations, the cumulative results were compared (Table 3). The results showed no significant difference between the resistance levels of the two populations against DDT ( $P > 0.05$ ,  $df = 37$ ,  $t = 1.032$ ) (Figure 9). However, the resistance ratio for the deltamethrin showed a significant difference between *Wolbachia* infected and uninfected populations ( $P < 0.05$ ,  $df = 36$ ,  $t = 3.950$ ). The untreated population showed lower resistance compared to the population which was treated against deltamethrin (Figure 10).

#### 4. Discussion

The relationship between insecticide resistance and the presence of *Wolbachia* bacteria in the host is one of the important issues in vector control, specially mosquitoes. In the context of parasitism and its relationship with the cost of resistance in mosquitoes to insecticides, Agnew et al. (2004) showed that *Vavraia culicis* can modify the costs of

organophosphate insecticide resistance in *Cx. quinquefasciatus*. In this study, we hypothesized that the presence of *Wolbachia* alters the response of *Cx. quinquefasciatus* to DDT and deltamethrin. Although many research have been done in this regard, there are still many unanswered questions about this relationship. Until now, the studies have not shown a clear effect of *Wolbachia* on insecticide resistance in mosquitoes. Endersby and Hoffmann (2012) examined the susceptibility levels in *Wolbachia* infected and uninfected populations of *Aedes aegypti* against bifenthrin, *Bacillus thuringiensis*, temephos, and s-methoprene. According to the results, there was no significant difference between the presence and absence of bacteria and resistance level in *Ae. aegypti* to insecticides. This outcome is in agreement with the result of our study concerning the response of *Cx. quinquefasciatus* to DDT in *Wolbachia* infected and uninfected populations.

Regarding other endosymbionts, the outcomes of studies have illustrated that, they usually cause higher susceptibility in their hosts rather than resistance (Li et al., 2020; Liu and Guo, 2019). In this respect, Kontsedalov et al. (2008) evaluated the resistance level in two *Rickettsia* infected and uninfected populations of *Bemisia tabaci* against six insecticides. They showed that although two populations did not differ in susceptibility to two of them, more susceptibility was observed in *Rickettsia* infected population against other insecticides. They concluded that in the presence of a symbiont the level of susceptibility to insecticides in *B. tabaci* increased. In 2009, Ghanim and Kontsedalov conducted another study on *B. tabaci* biotype Q. In this research, they evaluated the susceptibility level of three groups of *B. tabaci*, harboring different microorganisms against the insecticides mentioned in the previous study. The results suggested that the larger the bacterial density of the host, the more it is likely to detoxify insecticides. The results of another study by Skaljic et al. (2018) demonstrated that *Serratia* infection increased the susceptibility of pea aphid to four out of five insecticides. We achieved similar outcome related to deltamethrin. Based on our result, the *Wolbachia* infected population showed less resistance to deltamethrin compared to treated population. The different function of a symbiont may be associated with host species, symbiont species, chemicals, symbionts' density and the gene expression. In 2002, a study was conducted by Berticat et al. to find out the association between the presence of *Wolbachia* bacteria in the host and resistance genes. The results showed a significant change in bacterial density in the presence of resistance genes. They assumed that mosquitoes with resistance genes were not able to control the bacterial load in their bodies. As a result, this high volume of bacteria have virulent effects for the host. As it was observed in this study, *Wolbachia* infected population showed less resistance against deltamethrin which could be due to the same negative effect of the bacteria mentioned in Berticat et al. (2002). The negative effect of symbionts on their host fitness may increase their susceptibility. Skaljic et al. (2018) stated that fitness costs including lower reproduction and body weight in *Acyrtosiphon pisum* is due to the presence of a symbiont, which leads to higher susceptibility to insecticides.

In a study on *Wolbachia* density and its relationship with cost of infection in insecticide resistant *Cx. pipiens*, Duron et al. (2006)

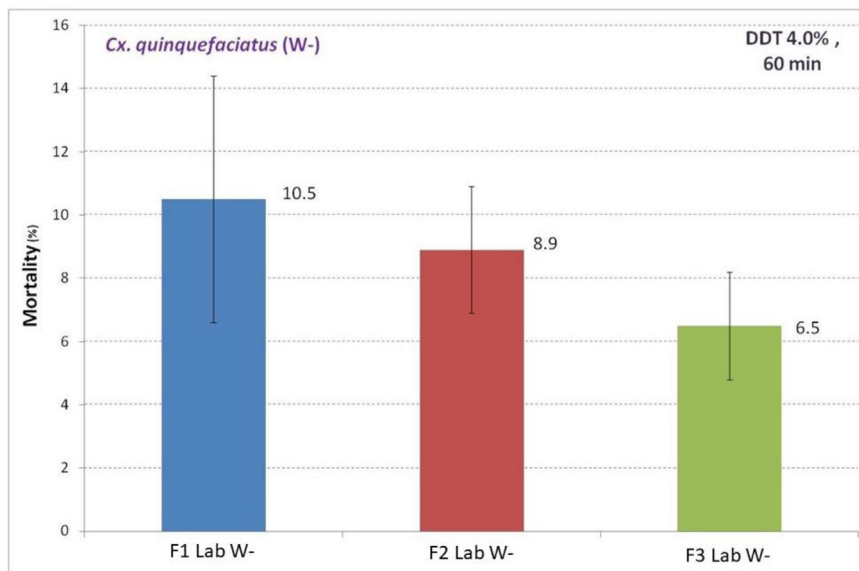


Figure 7. Comparison of susceptibility levels of different *Cx. quinquefasciatus* populations to DDT 4.0%.

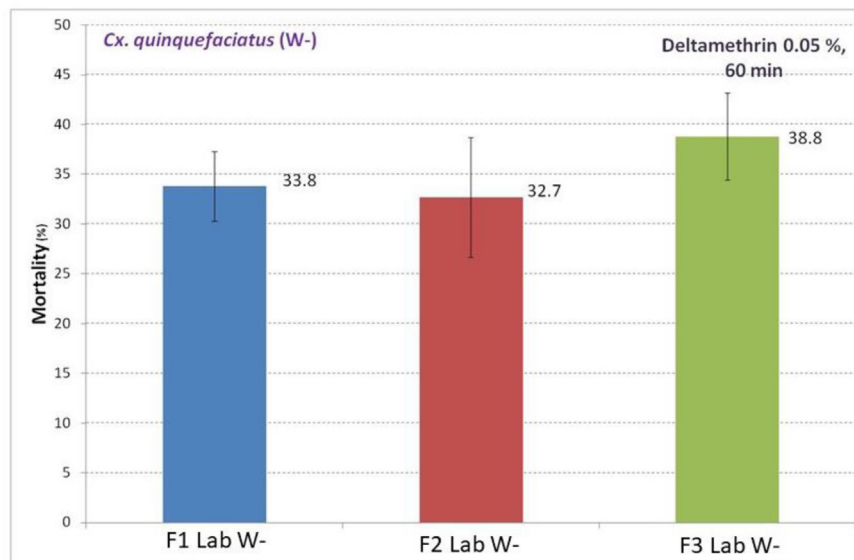


Figure 8. Comparison of susceptibility levels of different *Cx. quinquefasciatus* populations to deltamethrin 0.05%.

Table 2. Susceptibility levels to DDT 4.0% and deltamethrin 0.05% in three generations of *Wolbachia* negative *Cx. quinquefasciatus*.

Generation	Insecticide	No dead	No tested	Mortality ± SE
F1 Lab W-	DDT 4.0%	16	153	10.5 ± 3.9
	deltamethrin 0.05%	45	133	33.8 ± 3.5
	Control	0	64	0
F2 Lab W-	DDT 4.0%	15	168	8.9 ± 2
	deltamethrin 0.05%	49	150	32.7 ± 6
	Control	0	41	0
F3 Lab W-	DDT 4.0%	9	139	6.5 ± 1.7
	deltamethrin 0.05%	54	139	38.8 ± 4.4
	Control	0	43	0

Table 3. Comparison of susceptibility levels to DDT 4.0 % and deltamethrin 0.05% between treated and untreated populations of *Cx. quinquefasciatus*.

Insecticide	W+/W-	No dead	No tested	Mortality±SE
DDT	W+	59	491	12 ± 4.6
	W-	66	460	14.3 ± 2.5
deltamethrin	W+	258	503	51.3 ± 4.6
	W-	148	422	35.1 ± 4.6

discovered that bacterial density was higher in resistant mosquitoes. In order to determine whether resistance costs are due to resistance genes or high volume of the bacteria, they compared some strains of mosquitoes

with the same genetic background but with differences in resistance alleles and bacterial contamination status. The results showed that although *Wolbachia* bacteria are partly responsible for the fitness cost of insecticide resistance, there is still an association between fitness cost and resistance genes even in *Wolbachia* free population. Thus, for some uninfected resistant strains, the mortality rate remains high. Therefore, *Wolbachia* bacteria induce additional resistance costs for some specific traits. This result matches our finding regarding higher mortality in *Wolbachia*

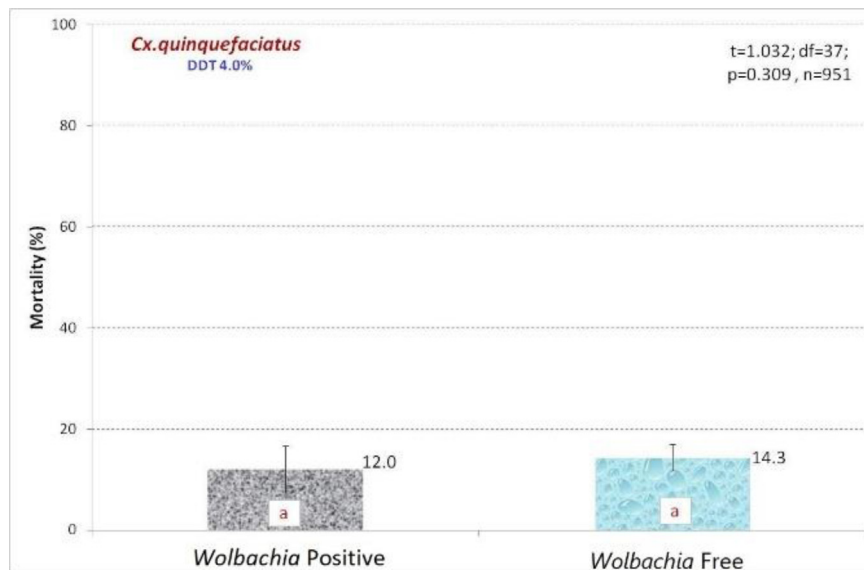


Figure 9. *Cx. quinquefasciatus* resistance level against DDT 4.0 % in *Wolbachia* infected and uninfected populations.

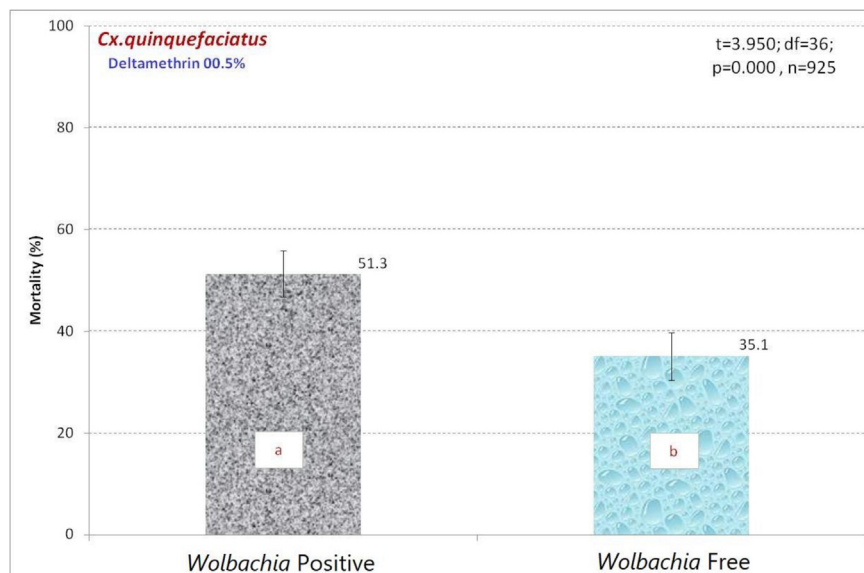


Figure 10. *Cx. quinquefasciatus* resistance level against deltamethrin 0.05% in *Wolbachia* infected and uninfected populations.

infected population against deltamethrin. In the case of insecticide resistant strain SA2, also, the authors did not observe any difference in resistance level in the presence and absence of bacteria. The outcome of the present study indicated that *Cx. quinquefasciatus* infected and uninfected populations showed no difference in the resistance level against DDT.

Based on the results of the present study, we found that the presence of *Wolbachia* bacteria in *Cx. quinquefasciatus* may play a role in decrease resistance to deltamethrin while having no effect on resistance to DDT. This means that *Wolbachia* have no adverse effect on chemical control of *Cx. quinquefasciatus* and does not exacerbate the resistance caused by using DDT and deltamethrin against this species. The result of this study may help with better understanding of the possible role of endosymbionts in resistance to insecticides, although more experiments in this area are still required.

#### Declarations

##### Author contribution statement

Atieh Shemshadian: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Hassan Vatandoost, Navid Dinparast Djavid: Conceived and designed the experiments.

Mohammad Ali Oshaghi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Mohammad Reza Abai: Conceived and designed the experiments; Analyzed and interpreted the data.

Fateh Karimian: Analyzed and interpreted data.

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##### Data availability statement

Data will be made available on request.

##### Declaration of interests statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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