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First report of the association between *Wolbachia* and *Cotesia flavipes* (Hymenoptera: Braconidae): effect on life history parameters of the parasitoid

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Research Paper

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Abstract

The symbiosis between microorganisms and host arthropods can cause biological, physiological, and reproductive changes in the host population. The present study aimed to survey facultative symbionts of the genera *Wolbachia*, *Arsenophonus*, *Cardinium*, *Rickettsia*, and *Nosema* in *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae) and *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) in the laboratory and evaluate the influence of infection on the fitness of these hosts. For this purpose, 16S rDNA primers were used to detect these facultative symbionts in the host species, and the hosts' biological and morphological features were evaluated for changes resulting from the infection caused by these microorganisms. The bacterial symbionts studied herein were not detected in the *D. saccharalis* samples analysed, but the endosymbiont *Wolbachia* was detected in *C. flavipes* and altered the biological and morphological aspects of this parasitoid insect. The results of this study may help to elucidate the role of *Wolbachia* in maintaining the quality of populations/lineages of *C. flavipes*.

Introduction

The sugarcane borer *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) is a polyphagous pest of several host Poaceae species that commonly affects sugarcane plantations throughout the American continent, mainly in Brazil, the world's largest sugarcane producer (Rossato *et al.*, 2013; Ferreira *et al.*, 2018). *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae) is the most widely used biological control agent for population regulation of *D. saccharalis.* In Brazil, *C. flavipes* is used on approximately 3.5 million hectares for the control of sugarcane borer, representing one of the most efficient applied biological control programmes in the world (Parra and Coelho, 2019; Fontes *et al.*, 2020). This successful control of *D. saccharalis* using *C. flavipes* shows the importance of measures to maintain the quality of this biological control agent and its host during the production process. Several factors can affect the fitness of insects; association with symbiotic organisms, for instance, has great ecological and evolutionary consequences for host species (Harris *et al.*, 2010; Dicke *et al.*, 2020).

Symbiosis is a broad term used to define the association between two or more species, and the effect of the symbiont on the host may be beneficial (mutualism), neutral (commensalism), or harmful (parasitism) (de Bary, 1879). Insects are natural hosts of numerous symbiotic microorganisms, and this association may have obligatory ecological and biological functions essential for survival – or facultative – often infecting only part of the population, being maintained through the provision of conditional benefits, or by manipulating the host's reproduction. The elimination of facultative symbionts often results in little or no apparent cost or benefit to the host insect (Douglas, 1989; Duron *et al.*, 2008; Brownlie and Johnson, 2009).

Symbiotic interactions between polydnaviruses and *Cotesia* spp. are commonly reported, with the former acting as important immune suppressors, allowing the development of immature parasitoids within the host (Stoltz and Vinson, 1979; Herniou *et al.*, 2013; Cônsoli and Kitajima, 2017; Tan *et al.*, 2018). Infection by the intracellular parasite *Nosema* sp. (Microspora: Nosematidae) has also been reported in *D. saccharalis* artificial rearings, and it affects the use of *C. flavipes* as a biological control by altering its biological parameters and search behaviour (Simões *et al.*, 2012). This shows the relevance of understanding symbiotic interactions and the effect of these microorganisms on the biological and morphological aspects of the association between *C. flavipes* populations. Thus, the objective of the present study was to identify facultative symbionts of the genera *Wolbachia*, *Arsenophonus*,

Cardinium, *Rickettsia*, and *Nosema* in *C. flavipes* and the host *D. saccharalis*, as well as to evaluate the influence of their infection on the fitness of these hosts.

Materials and methods

Breeding and bioassays were conducted under controlled conditions at a temperature of 25 ± 1°C, relative humidity of 60 ± 10%, and a 12 h photophase in the laboratories of the Department of Crop Protection, São Paulo State University, School of Agronomic Sciences, Botucatu, São Paulo, Brazil.

Obtaining and multiplying insects

The *D. saccharalis* and *C. flavipes* individuals used in the assays were obtained from the São Paulo biofactory, Brazil. *Diatraea saccharalis* were fed an adapted version of the artificial diet proposed by Hensley and Hammond (1968) (agar was replaced with carrageenan), and breeding methodologies for both species followed those of Garcia *et al.* (2009).

Detection of symbionts

Genomic DNA extraction

Larvae of the host *D. saccharalis* and adults of *C. flavipes* (*n* 50 for each species) were randomly selected from the breeding material stored in the AGRIMIP laboratory (FCA/UNESP) for genomic DNA extraction and standardisation via polymerase chain reaction (PCR). The *D. saccharalis* larvae were washed with saline solution (0.85% NaCl) followed by 70% alcohol and subsequently macerated in a sterile 10 ml glass beaker, after which parts of the insect's body were removed. The remaining body content was dissolved in 80 µl of Chelex100 resin (Bio-Rad Laboratories, California, US) at 10%, diluted in sterile water, and dissolved in 8 µl of proteinase K (20 µg ml^{-1}) in 200 µl microtubes. For DNA extraction from *C. flavipes*, a similar procedure was performed, except that all adults of *C. flavipes* individuals were directly macerated in 200 µl microtubes, without removing parts of the insect's body. All tubes containing the larvae of *D. saccharalis* and the adults of *C. flavipes* were then vortexed for 5 s in a Vortex Biomixer MOD QL901, centrifuged at 6200 rpm in a MiniStar mini centrifuge, and transferred to an Infinigen thermocycler (TC-96CG) at 95°C for 20 min.

Polymerase chain reaction (PCR)

PCR and mass sequencing amplification targeting the small subunit region of ribosomal RNA were performed using specific primers for symbionts of the genera *Wolbachia*, *Arsenophonus*, *Cardinium*, *Rickettsia*, and *Nosema* (table 1). For the detection of all the genera except *Nosema*, the PCR mixture contained 12.5 µl of Taq DNA Polymerase (NeoBio), 7.5 µl of milli-Q water, 1.0 µl of each primer, and 3.0 µl of DNA sample, for a total volume of 25 µl. For *Nosema*, the PCR mixture contained 12.5 µl of Taq DNA Polymerase (NeoBio), 5 µl of milli-Q water, 1.25 µl of each primer, and 5.0 µl of DNA sample, for a total volume of 25 µl.

The PCRs were performed in an Infinigen thermocycler (model TC-96CG), under the following conditions: *Arsenophonus*, initial denaturation at 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min (Thao and Baumann, 2004); *Cardinium*, initial denaturation at 95°C for 2 min, followed by 30 cycles at 92°C for 30 s, 57°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min (Zchori-Fein and Perlman, 2004); *Rickettsia*, initial denaturation at 95°C for 2 min, followed by 30 cycles at 92°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min (Gottlieb *et al.*, 2006); *Wolbachia*, initial denaturation at 95°C for 3 min, followed by 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and final extension at 72°C for 5 min (Heddi *et al.*, 1999); *Nosema*, initial denaturation at 95°C for 4 min, followed by 45 cycles at 95°C for 1 min, 48°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 4 min (Vossbrinck *et al.*, 1993).

PCR products were read in a UV light transilluminator (Major Science) using a 100-bp molecular marker (Norgen) and a 1% agarose gel containing 80 ml of TBE buffer solution, 0.8 g of agarose (Neo3Bio), and 0.4 µl of GelRed DNA intercalant (NeoBio).

DNA purification and Sanger sequencing

PCR products in which symbionts were detected were purified using a Cellco purification kit, according to the manufacturer's recommendations. Quantitative analyses were performed by optical density and spectrophotometry (NanoDrop MD-1000 UV-Vis). The amplified fragments were sequenced using an automatic Sanger sequencer (Model: ABI 3500, Applied Biosystems) at the Biotechnology Institute (*Instituto de Biotecnologia*, IBTEC) of UNESP, Botucatu, São Paulo, Brazil. The obtained sequences were compared and deposited in the GenBank database (National Center for Biotechnology Information, NCBI) using the Basic Local Alignment Search Tool (BLAST), and specific identification was performed based on sequence similarity scores and percentage of similarity.

Post-symbiont lineages production of Cotesia flavipes

After confirming infection by *Wolbachia* in *C. flavipes*, individuals were selected for the production of sister lineages without symbionts. Some of the individuals were fed pure honey to maintain the association with *Wolbachia* (W^+), while the other group was fed honey supplemented with antibiotics (0.25% tetracycline) to eliminate the symbiont (W^-) (Li *et al.*, 2014). The parasitoids (W^-) were maintained in a biological oxygen demand incubator at 28 ± 1°C under a relative humidity of 70 ± 10% and a 12 h photoperiod. The process of eliminating *Wolbachia* was conducted for four consecutive generations, after which individuals from both populations were randomly selected to confirm the absence of the symbiont using PCR, following the aforementioned methodologies. After this decontamination process, the W^- population was fed for ten generations with pure honey to allow a complete restoration of the intestinal microbiota and eliminate all side effects of the treatment.

Wolbachia infection in *C. flavipes* populations (W^+ and W^-) was investigated before and after the experiments to validate the results obtained, as the populations of the parasitoid and the host *D. saccharalis* were evaluated for infection by *Nosema* sp., as this microorganism is commonly reported in parasitoids that multiply in *D. saccharalis*, causing deleterious effects in the infected population (Simões *et al.*, 2012; Paes *et al.*, 2019)

Changes in the fitness of Cotesia flavipes *associated with* Wolbachia *infection*

Diatraea saccharalis larvae suitable for parasitism (4th instar) were selected from the breeding material stored at AGRIMIP

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Table 1. Primers used for detecting symbionts of *Diatraea saccharalis* (Lepidoptera: Crambidae) and *Cotesia flavipes* (Hymenoptera: Braconidae).

Symbiont	Target gene	Primer sequence 5′ > 3′	(pb)	References
Arsenophonus	*16S rRNA*	F-CGTTTGATGAATTCATAGTCAAA	600	Thao and Baumann (2004)
		R-GGTCCTCCAGTTAGTGTTACCCAAC		
Cardinium	*16S rRNA*	F-TACTGTAAGAATAAGCACCGGC	900	Zchori-Fein and Perlman (2004)
		R-GTGGATCACTTAACGCTTTCG		
Rickettsia	*16S rRNA*	F-GCTCAGAACGAACGCTATC	900	Gottlieb *et al.* (2006)
		R-GAAGGAAAGCATCTCTGC		
Wolbachia	*16S rRNA*	F-CGGGGGAAAAATTTATTGCT	700	Heddi *et al.* (1999)
		R-AGCTGTAATACAGAAAAGTAAA		
Nosema	*16S rRNA*	F-CACCAGGTTGATTCTGCC	222	Vossbrinck *et al.* (1993)
		R-TTATGATCCTGCTAATGGTTC		

(FCA/UNESP) and placed with adult W^+ and W^- *C. flavipes* females (48 h old) to allow the occurrence of parasitism. Then, the larvae were individually placed in plastic capsules (3 × 7 cm, diameter × height) with a refeeding diet (Hensley and Hammond, 1968). Sixty larvae were replicated for each population of the parasitoid for evaluations of biological characteristics, flight capacity, and morphometry.

Biological aspects

The characteristics of *C. flavipes* were evaluated after the parasitoid larvae exited the body of *D. saccharalis* and during pupation. Twenty pupal masses from each population (W^+ and W^-) were then removed, placed in glass tubes (2.5 × 1 cm, diameter × height), and observed daily to determine the egg–pupa and pupa–adult development period (days), pupal viability (equation 1), female proportion (equation 2), and adult survival.

$$\text{Viability (\%)} = \frac{\text{Total number of pupae}}{\text{Number of parasitoids}} \times 100 \quad (1)$$

$$\text{Female proportion (\%)} = \frac{\text{Number of females}}{\text{Number of females} + \text{Number of males}} \times 100 \quad (2)$$

The survival of adult males and females was evaluated by randomly selecting one individual of each sex from each replicate, totalling 20 individuals of both sexes, placing them in individual glass tubes (2.5 × 8 cm, diameter × height), and observing them daily until death. Adults were fed thin lines of pure honey and inserted into these tubes using an entomological pin.

Flight capacity

The flight capacity of W^+ and W^- *C. flavipes* was evaluated following the methodology of Dutton and Bigler (1995) and adapted by Prezotti *et al.* (2002). The test units for flight capacity consisted of a PVC cylinder internally covered with black cardboard and with the bottom sealed with black paper adjusted on a 1 cm-thick Styrofoam disk with the same diameter as the cylinder. In the test unit, an entomological glue ring was painted over an acetate strip (1 cm thick) 3.5 cm from the lower end of the cylinder, acting as a barrier for walking parasitoids. The upper part of the test unit was sealed with a transparent Petri dish internally covered with entomological glue, which acted as a trap for the parasitoids in flight.

A glass tube (2.5 × 8 cm, diameter × height) containing a pupa mass with approximately 100 ready-to-emerge *C. flavipes* pupae was fixed at the centre of the bottom of the test unit, on the Styrofoam disk. Inside the tube, droplets of pure honey were provided as food for the parasitoids. A total of 20 test units were used for each parasitoid population (W^+ and W^-), which were maintained in a vertical laminar flow chamber under fluorescent light for 3 days. After this period, the number of *C. flavipes* specimens in the glue ring (walkers), the Petri dish (flyers), and the bottom of the cylinder (non-flyers) was determined.

Morphometry

Ten W^+ and W^- adult females and ten W^+ and W^- males were measured. The parasitoids were placed on slides containing alcohol gel, positioned in a right-side view, and photographed using a Leica EZ4 D optical microscope coupled to a camera. The following structures were measured in ImageJ 2.00: (1) total length (thorax + abdomen); (2) length of the right forewing; (3) width of the right anterior wing; and (4) length of the posterior tibia (from the junction of the tibia with the tarsus).

Statistical analysis

The data resulting from the tests were assessed through exploratory analyses for an evaluation of the assumptions of normality and homogeneity of variances using the Shapiro–Wilk ($P < 0.05$) and Bartlett ($P < 0.05$) tests, respectively. The egg–pupa and adult–pupa development periods, viability, female proportion, flight test results, and morphometric data were analysed by *t* tests ($P < 0.05$). The differences in flight capacity among the categories (flying, walking, and non-flying insects) were evaluated by analysis of variance and compared by Tukey tests ($P < 0.05$). Kaplan–Meier survival curves were generated from survival data and compared using the logRank test ($P < 0.05$). All analyses were performed using Minitab software.

Results

Diatraea saccharalis *and* Cotesia flavipes *symbionts*

In the present study, no associations between the studied symbiont species and *D. saccharalis* were found. However, the

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Table 2. Development (days) of egg–pupa and adult–pupa, viability (%), and female proportion of *Cotesia flavipes* (Hymenoptera: Braconidae) infected (W^+) and not infected (W^-) with *Wolbachia*

Biological characteristics	*Cotesia flavipes* (W^+)	*Cotesia flavipes* (W^-)
Development (days) of egg–pupa	12.82 ± 0.09^{ns}	12.50 ± 0.14
Development (days) of adult–pupa	6.11 ± 0.36^{ns}	6.07 ± 0.17
Viability (%)[a]	83.81 ± 1.61^{ns}	73.78 ± 3.30
Female proportion (%)[b]	56.00 ± 0.02^{ns}	54.00 ± 0.03

The experimental conditions were: 25 ± 1°C, relative humidity of 60 ± 10%, and a 12 h photophase.
[a]Calculated according to equation 1.
[b]Calculated according to equation 2.
Means different letters within indicate significant difference (*t* test, $P < 0.05$).

α-proteobacterium *Wolbachia* was detected in *C. flavipes* (GenBank accession number: OR074180), with a sequencing coverage of 98% identity for *Wolbachia* (closest GenBank accession: CP037426.1)

Changes in the fitness of Cotesia flavipes *associated with* Wolbachia *infection*

Biological aspects

No significant differences were detected in the egg–pupal development period (days) ($t = 1.897$, df = 1, $P = 0.061$; table 2), adult–pupal development period ($t = 0.193$, df = 1, $P = 0.847$; table 2), pupal viability ($t = -0.658$, df = 1, $P = 0.512$; table 2), or female proportion ($t = 0.468$, df = 1, $P = 0.641$; table 2) between the two populations.

Survival analysis revealed no difference in the longevity of males between the two populations, with a mean survival of 48 h ($\chi^2 = 0.190$, df = 1, $P = 0.663$; fig. 1b). However, significant differences were observed in the survival of females in the two populations ($\chi^2 = 11.598$, df = 1, $P < 0.001$; fig. 1a). Females without *Wolbachia* had a mean survival of 72 h, which was longer than that of females with the symbiont (48 h) (fig. 1a).

Flight capacity

No significant differences were observed in the percentages of the flyer ($t = -0.89$, df = 1, $P = 0.3776$; fig. 2), walker ($t = 0.75$, df = 1, $P = 0.4587$; fig. 2), and non-flyer ($t = -0.85$, df = 1, $P = 0.4749$; fig. 2) adults between the W^+ and W^- populations (fig. 2). However, the number of flyers was greater than that of walkers and non-flyers in all populations (W^+: $F = 138.22$, df = 2, $P \leq 0.0001$; W^-: $F = 233.00$, df = 2, $P \leq 0.0001$; fig. 2).

Morphometry

No significant differences were detected in body length ($t = -1.169$, df = 1, $P = 0.258$; fig. 3) or right tibia length ($t = -1.482$, df = 1, $P = 0.156$; fig. 3) between the female *C. flavipes* W^+ and W^- populations. However, compared with W^+ females, *C. flavipes* W^- females had longer right-wing lengths ($t = -2.449$, df = 1, $P = 0.025$) and wider right-wing lengths ($t = -2.742$, df = 1, $P = 0.013$) (fig. 3).

Uninfected males of *C. flavipes* (W^-) had a longer right tibia length than individuals infected with *Wolbachia* (W^+) ($t = -2.70$, df = 1, $P = 0.015$; fig. 4). No significant differences were detected in the other morphological measures between males of the two populations (body length: $t = 0.285$, df = 1, $P = 0.779$; right-wing length: $t = -1.820$, df = 1, $P = 0.085$; right-wing width: $t = -1.799$, df = 1, $P = 0.089$; fig. 4).

Discussion

Symbiotic associations between *Wolbachia* and *Cotesia* spp. have already been reported in the literature, but the associations between *Wolbachia* and *C. flavipes* are presented herein for the first time (Mochiah *et al.*, 2002; Branca *et al.*, 2011, 2019; Rattan *et al.*, 2011; Srinivasa *et al.*, 2011; Murthy *et al.*, 2015). *Wolbachia* is a genus of Gram-negative, non-spore-forming, obligate intracellular symbiont bacteria that infects a wide range of arthropods. *Wolbachia* can be transmitted vertically from host females to offspring by being loaded into the egg (O'Neill *et al.*, 1997; Duron *et al.*, 2008). Some studies have also reported the horizontal transfer of *Wolbachia* across populations (O'Neill *et al.*, 1992; Werren *et al.*, 1995). Nevertheless, as no infections

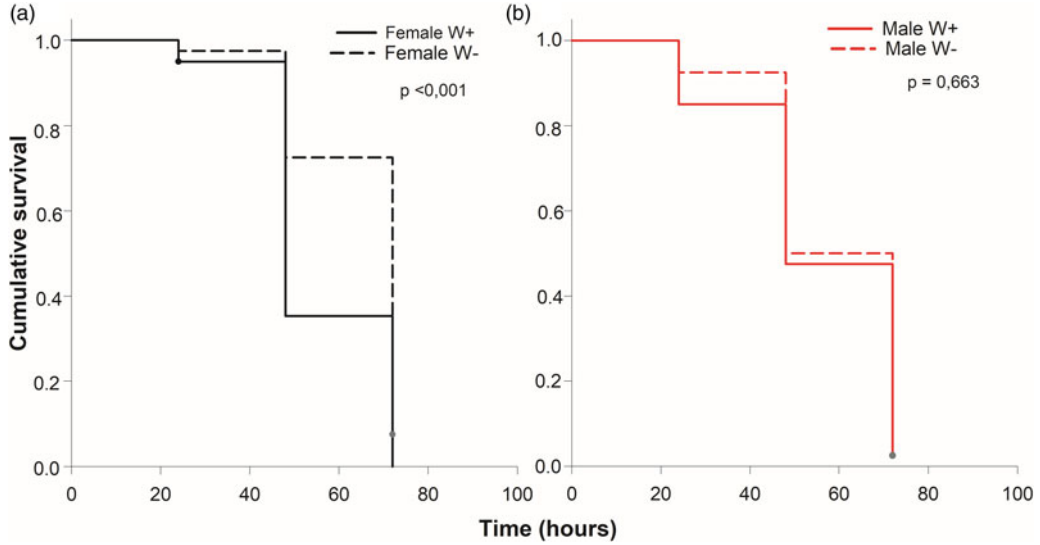

Figure 1. Survival *of Cotesia flavipes* (Hymenoptera: Braconidae) infected (W^+) and not infected (W^-) with *Wolbachia*. Females (W^+ and W^-) (A) and males (W^+ and W^-) (B). The experimental conditions were: 25 ± 1°C, relative humidity of 60 ± 10%, and a 12 h photophase (LogRank test, $P < 0.05$).

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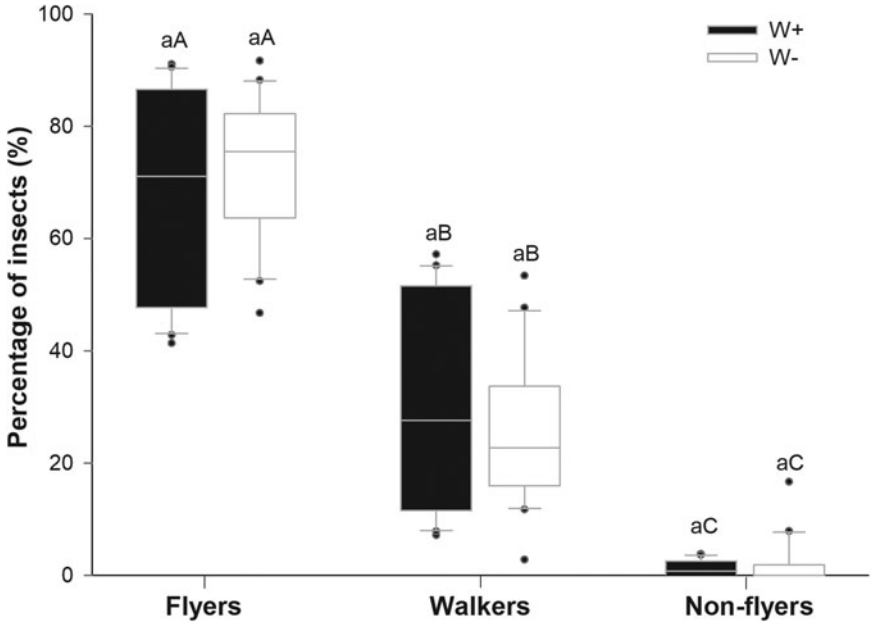

Figure 2. Percentages of flyer, walker, and non-flyer adults of *Cotesia flavipes* (Hymenoptera: Braconidae) previously infected (W^+) and not infected (W^-) by *Wolbachia*. Box plots represent the median and median quartiles of 75 and 25%, whiskers represent upper and lower bounds, and dots represent value discrepancies. The experimental conditions were: 25 ± 1°C, relative humidity of 60 ± 10%, and a 12 h photophase. Columns with the same lowercase letter (among populations) and capital letter (within population) are not significantly different from each other (*t* test, $P < 0.05$ and Tukey test, $P < 0.05$, respectively).

of this symbiont were observed in *D. saccharalis*, *Wolbachia* transmission in *C. flavipes* was herein demonstrated to occur vertically.

Wolbachia infection mainly affects the host's ecological aspects and reproductive system, inducing cytoplasmic incompatibility, parthenogenesis, feminisation, and annihilation of males, which results in reproductive isolation of the infected population and, consequently, successful establishment of the symbionts in host population (Stouthamer *et al.*, 1999; Mochiah *et al.*, 2002). Although some *Wolbachia* strains successfully infect host populations, others cannot parasitise the reproductive systems of their

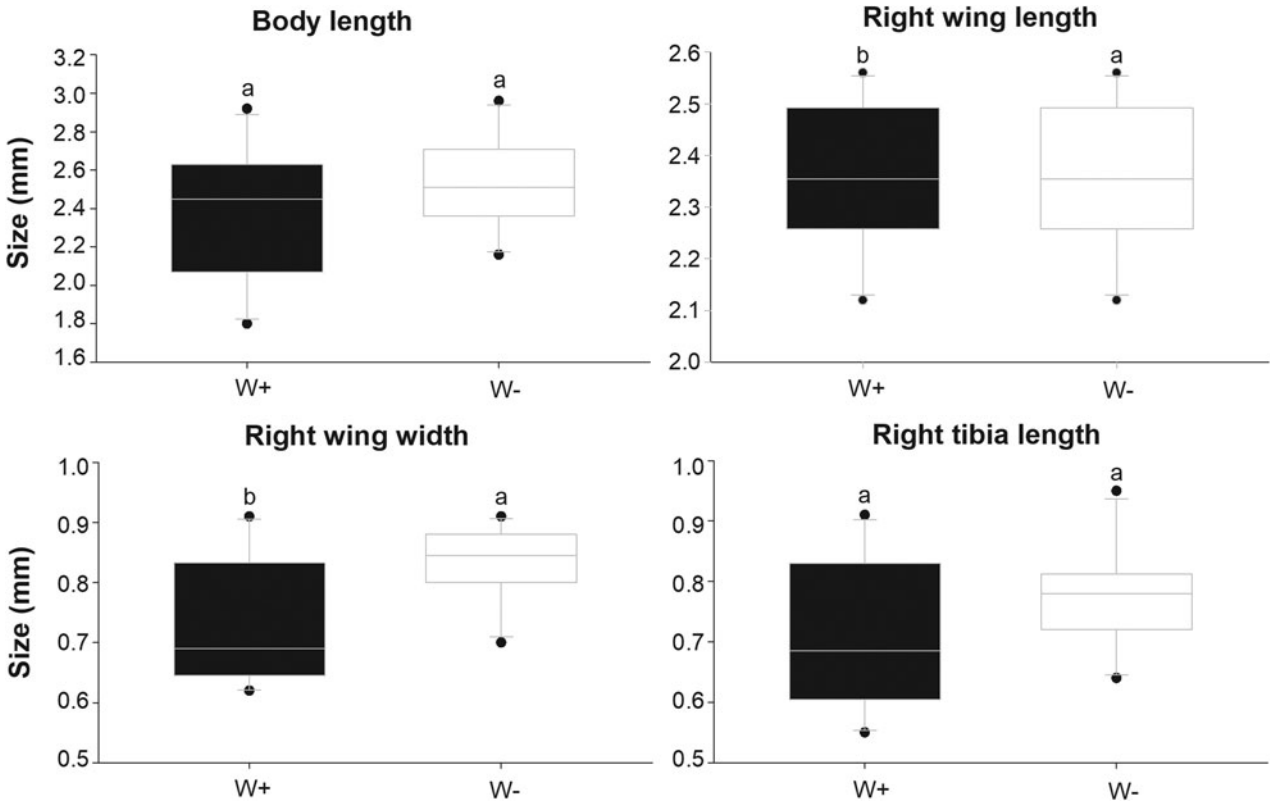

Figure 3. Body length, right-wing length, right-wing width, and right tibia length (all in mm) of females of *Cotesia flavipes* (Hymenoptera: Braconidae) infected (W^+) (black boxes) and not infected (W^-) (white boxes) with *Wolbachia*. Box plots represent the median and median quartiles of 75 and 25%, respectively; whiskers represent upper and lower bounds, and dots represent outliers. The experimental conditions were: 25 ± 1°C, a relative humidity of 60 ± 10%, and a 12 h photophase. The different letters within each graph indicate significant differences (*t* test, $P < 0.05$).

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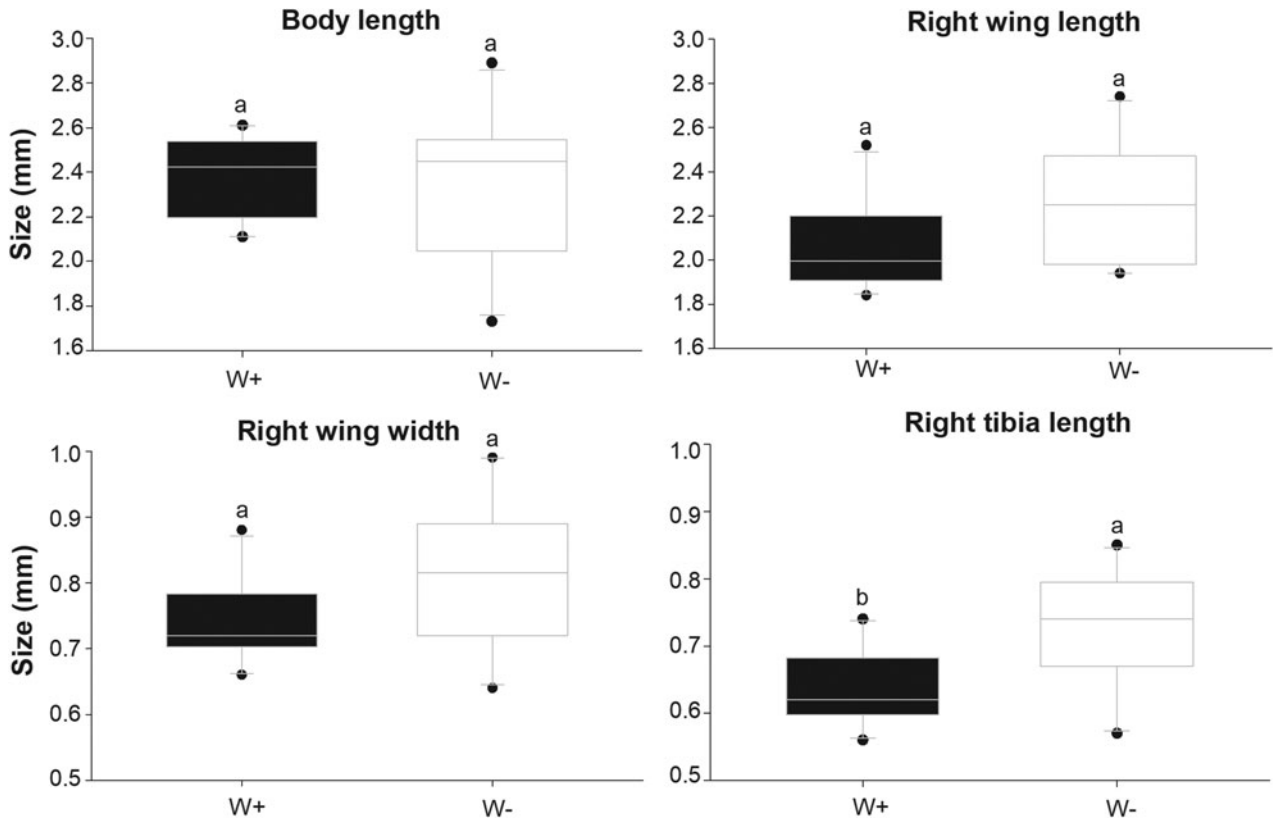

Figure 4. Body length, right-wing length, right-wing width, and right tibia length (all in mm) of males of *Cotesia flavipes* (Hymenoptera: Braconidae) infected (W^+) (black boxes) and not infected (W^-) (white boxes) with *Wolbachia*. Box plots represent the median and median quartiles of 75 and 25%, respectively; whiskers represent upper and lower bounds, and dots represent outliers. The experimental conditions were: 25 ± 1°C, a relative humidity of 60 ± 10%, and a 12 h photophase. The different letters within each graph indicate significant differences (*t* test, $P < 0.05$).

host or adequately do so (Hoffmann *et al.*, 1996). This may explain the findings of the present study, as feminisation and annihilation of males infected by *Wolbachia* were not observed herein, suggesting that this symbiont cannot induce thelytokous parthenogenesis in this population and/or cannot replicate at high levels in the body of this parasitoid. Although *Wolbachia*-induced parthenogenesis in *C. flavipes* was not observed herein, future investigations should evaluate the effect of bacterial infection on the intraspecific cytoplasmic incompatibility of this insect; a mixture of infected and uninfected populations with such reproductive changes would have a decreased population growth rate, which would have implications for biological control programmes.

The present study also detected possible deleterious effects of the symbiotic association on the biological and morphological aspects of *C. flavipes*. Uninfected females had longer right-wing lengths and wider right-wing lengths and were longer lived, and uninfected males had longer tibia lengths than males infected with *Wolbachia*. The results presented here are supported by several reports of deleterious effects on the fitness of arthropods infected with *Wolbachia* (Fry *et al.*, 2004; McGraw and O'Neill, 2004; Serga *et al.*, 2014; Stevanovic *et al.*, 2015; Zhou *et al.*, 2023).

Considering that the longevity and morphology of parasitoids are considered indicators of the quality of these control agents (Sagarra *et al.*, 2001; Wang and Keller, 2020), the information from this study is the basis for understanding the impact of costs of *Wolbachia* infection in *C. flavipes*; therefore, measures are needed to investigate infections by this symbiont in large-scale production of *C. flavipes* to maintain the production of efficient individuals for use in augmentative biological control programmes.

Such effects on the fitness of arthropods can be reversed by treating the infected population with antibiotics, as observed in *C. flavipes*. Dedeine *et al.* (2001, 2005) reported the participation of this bacterium in the oogenesis of *Asobara tabida* Nees (Hymenoptera: Braconidae), with post-symbiont females being unable to produce mature oocytes and, therefore, reproducing; this was the first record of a transition from facultative to obligatory symbiosis in arthropod associations. Nevertheless, no such effect of *Wolbachia* infection was found herein, as the post-symbiont *C. flavipes* populations showed no reproductive changes.

Conclusions

The results of the present study can be used as a basis for understanding the role of *Wolbachia* in the quality of *C. flavipes* populations, in addition to demonstrating the importance of studies on these microorganisms that influence their host's biological, physiological, and reproductive characteristics.

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