

# Antiviral Protection and the Importance of *Wolbachia* Density and Tissue Tropism in *Drosophila simulans*

Sheree E. Osborne,<sup>a</sup> Iñaki Iturbe-Ormaetxe,<sup>c</sup> Jeremy C. Brownlie,<sup>b</sup> Scott L. O'Neill,<sup>c,d</sup> and Karyn N. Johnson<sup>a</sup>

School of Biological Sciences, The University of Queensland, St. Lucia, Queensland, Australia<sup>a</sup>; School of Biomolecular and Physical Sciences, Griffith University, Brisbane, Queensland, Australia<sup>b</sup>; School of Biological Sciences, Monash University, Victoria, Australia<sup>c</sup>; and Institute of Molecular Biosciences, The University of Queensland, St. Lucia, Queensland, Australia<sup>d</sup>

*Wolbachia*, a maternally transmitted endosymbiont of insects, is increasingly being seen as an effective biological control agent that can interfere with transmission of pathogens, including dengue virus. However, the mechanism of antiviral protection is not well understood. The density and distribution of *Wolbachia* in host tissues have been implicated as contributing factors by previous studies with both mosquitoes and flies. *Drosophila* flies infected with five diverse strains of *Wolbachia* were screened for the ability to mediate antiviral protection. The three protective *Wolbachia* strains were more closely related and occurred at a higher density within whole flies than the two nonprotective *Wolbachia* strains. In this study, to further investigate the relationship between whole-fly *Wolbachia* density and the ability to mediate antiviral protection, tetracycline was used to decrease the abundance of the high-density, protective *Wolbachia* strain wAu prior to viral challenge. Antiviral protection was lost when the density of the protective *Wolbachia* strain was decreased to an abundance similar to that of nonprotective *Wolbachia* strains. We determined the *Wolbachia* density and distribution in tissues of the same five fly-*Wolbachia* combinations as used previously. The *Wolbachia* density within the head, gut, and Malpighian tubules correlated with the ability to mediate antiviral protection. These findings may facilitate the development of *Wolbachia* biological control strategies and help to predict host-*Wolbachia* pairings that may interfere with virus-induced pathology.

*Wolbachia*, an intracellular endosymbiont of insects, is increasingly being seen as an effective biological control agent that can interfere with the transmission of pathogens that are vectored by insects (7, 13, 14, 19, 23, 34, 38, 42, 43). *Wolbachia* is a maternally transmitted bacterium which is predicted to infect up to 70% of insect species (17, 24, 45). *Wolbachia* bacteria are best known for their ability to manipulate host reproductive systems, leading to an increase in their frequency within insect populations over successive generations (20, 35, 40, 44). *Wolbachia* infection can also reduce the ability of insects to transmit viruses such as dengue and Chikungunya viruses and can protect the insect against viral, bacterial, and protozoan parasites (2, 12, 15, 22, 26, 27, 34, 36, 39). However, the mechanism by which *Wolbachia* protects against pathogens is not well understood. A better understanding of endosymbiont characteristics and antipathogen protection may facilitate the development of improved strategies for use of *Wolbachia* as a biocontrol agent.

*Wolbachia* endosymbiosis has been well characterized within the model organism *Drosophila*, in which protection against viral challenge has been observed (15, 36, 39). *Wolbachia*-mediated antiviral protection has been described to occur in *Drosophila melanogaster* and *Drosophila simulans*, flies which exhibit delayed mortality when challenged with RNA viruses, including *Drosophila C* virus (DCV), cricket paralysis virus, Flock House virus, and Nora virus (15, 36, 39). In some *Wolbachia*-host combinations, a delay in the accumulation of viral particles was observed concurrently with a delay in mortality (15, 36, 39). Interestingly, some *Wolbachia*-containing flies, such as the *D. simulans* CO line which contains the *Wolbachia* strain wAu (CO-wAu), were better able to tolerate high titers of virus than *Wolbachia*-free flies (36). It is not yet understood how the presence of *Wolbachia* delays virus accumulation or how it contributes to host tolerance of viral infection. Furthermore, not all *Wolbachia*-fly pairings are protected against

virus-induced mortality (29, 36). When five *D. simulans*-*Wolbachia* combinations were screened for antiviral protection by viral challenge, three combinations were protected (CO-wAu, Me29-wMel, and DSR-wRi), whereas the other two were not (DSH-wHa and N7NO-wNo) (36). Notably, when the same fly combinations were challenged with mortality-inducing bacteria, including *Erwinia carotovora*, none of the *Wolbachia* strains mediated antibacterial protection (47). The difference in response between antiviral and antibacterial protection in the same fly-*Wolbachia* combination indicates that the *Wolbachia*-mediated mechanism of antiviral protection differs from the mechanism of antibacterial protection (47).

There are a number of *Wolbachia*-virus, or *Wolbachia*-host-virus, interactions that could result in antiviral protection. Examples of these mechanisms could include competition between *Wolbachia* and virus for a shared resource, steric hindrance between *Wolbachia* and the virus within the internal cellular environment, altered cell membrane characteristics, or altered cell behaviors such as viral uptake. Limited data are available on the influence of *Wolbachia* on these factors. If these factors contribute to host susceptibility to virus, the abundance of *Wolbachia* would likely impact the ability to mediate antiviral protection. *Wolbachia* density may be important at a cellular level, at the tissue level, or within the whole host organism (10, 34, 36). For example, partic-

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Address correspondence to Karyn N. Johnson, [karynj@uq.edu.au](mailto:karynj@uq.edu.au).

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ular tissues may play a role in the host's viral response or be relevant to viral pathogenesis. If viral pathogenesis relies on the infection of a particular cell or tissue type and *Wolbachia* can limit or modulate viral infection within that tissue, the outcome of virus-induced disease may be modified. Although the majority of *Wolbachia* distribution studies have focused on the reproductive organs, *Wolbachia* has also been identified in a number of somatic tissues across insect species (4, 5, 8, 11, 32–34, 42). Previous studies used different *Wolbachia* strains, host backgrounds, and techniques to identify *Wolbachia* in tissue types (2, 4, 5, 8, 34). Intrinsic host and *Wolbachia* strain factors have been shown to alter *Wolbachia* distribution within tissues (8, 41). To our knowledge, no study has yet compared *Wolbachia* tissue distribution and density in natural host-*Wolbachia* combinations to identify tissues of potential importance in *Wolbachia*-mediated antiviral protection.

Data from previous studies suggest that *Wolbachia* density and distribution may be important for antiviral protection to occur (10, 34, 36). When diverse strains of *Wolbachia* in *D. simulans* host backgrounds were screened for the ability to mediate antiviral protection, a correlation was identified between *Wolbachia* density in whole flies and the ability to mediate antiviral protection. The *Wolbachia* strains that mediated antiviral protection (*wAu*, *wMe29*, and *wRi*) occurred at a higher abundance within individual flies and were more closely related than the *Wolbachia* strains that did not mediate antiviral protection (*wHa* and *wNo*) (36, 48). The importance of *Wolbachia* density has also been implicated in mosquito and mosquito cell line studies (10, 34). This study investigated the importance of *Wolbachia* density and distribution within the host on the ability of the strain to mediate antiviral protection across the five fly-*Wolbachia* combinations. Then the abundance of the protective *wAu* strain was decreased and flies were challenged with the mortality-inducing DCV to determine if *Wolbachia* abundance is an important factor in antiviral protection.

## MATERIALS AND METHODS

***Drosophila* and *Wolbachia*.** Five *D. simulans* lines (CO, Me29, DSR, N7NO, and DSH) were maintained at 25°C with a 12-h light-dark cycle, on standard cornmeal agar media as previously described (36). For each fly line, paired populations of flies were used that either contained a *Wolbachia* strain (CO-*wAu*, Me29-*wMel*, DSR-*wRi*, N7NO-*wNo*, and DSH-*wHa*) or had been previously cured of *Wolbachia* (CO-T, Me29-T, DSR-T, N7NO-T, and DSH-T) by tetracycline treatment (21, 36). As tetracycline treatment may impact the fly gut microbiota, following tetracycline treatment the flies were placed on food that had been exposed to untreated male flies. With the exception of Me29-*wMel*, natural fly-*Wolbachia* combinations were used (18, 21, 31, 35). Me29 had previously been transinfected with the *D. melanogaster* *Wolbachia* strain *wMel*, by microinjection of infected cytoplasm from *D. melanogaster* Wein 5 embryos into Me29 NHaTC embryos (37).

**Preparation of *Drosophila* histological sections.** Wings and legs were removed from 4- to 7-day-old male anesthetized flies, and insects were preserved in 4% paraformaldehyde plus 0.5% Triton X-100 at 4°C for at least 2 days. Flies were dehydrated in 50% ethanol for 3 h, then 70% ethanol overnight, 90% ethanol for 1 h, and 95% ethanol for 1 h, and rinsed twice in 100% ethanol for 30 min. A 15-min rinse in toluene, followed by overnight storage in cedar wood oil and a 10-min rinse in 1:1 toluene-cedar wood oil, was used to improve wax infiltration. Samples were submerged in paraffin wax (Paraplast-Xtra; McCormick Scientific) at 60°C for 30 min and then twice for 15 min under vacuum, with 5 to 15 insects per wax mold. A rotary microtome was used to obtain 8- $\mu$ m-thick sections that were adhered to Super Frost Plus slides (Menzel-Gläser).

After a minimum of 24 h, slides were deparaffinated by three 3-min xylene washes and hydrated with a series of ethanol washes: 100% ethanol twice for 3 min, 90% ethanol for 3 min, 70% ethanol for 3 min, distilled water for 5 min, and two dips in distilled water. Slides were air dried and stored at room temperature until use.

**Fluorescent *in situ* hybridization (FISH).** Histological slides of flies with and without *Wolbachia* were treated with hybridization buffer which contained a final concentration of 4 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% (vol/vol) deionized formamide, 0.2 g/ml dextran sulfate, 0.25 mg/ml salmon sperm DNA, 0.25 mg/ml poly(A), 0.25 mg/ml tRNA, 0.1 M dithiothreitol (DTT), and 0.5 $\times$  Denhardt's solution. Two oligonucleotide probes labeled at the 3' end with rhodamine were used to target the 16S rRNA region of the *Wolbachia* species (34). BLAST (NCBI) alignment on the five *Wolbachia* strains indicated that both probes (5'-CTTCTGTGAGTACCGTCATTATC-3' and 5'-AACCG ACCCTATCCCTTCGAATA-3') were 100% homologous to all the strains used in this study. Slides were treated with 200  $\mu$ l of hybridization solution containing a final concentration of 15 nM for both probes and incubated overnight at 37°C. Slides were washed once in 1 $\times$  SSC and 10 mM DTT for 10 min, twice in 1 $\times$  SSC and 10 mM DTT at 55°C for 15 min, twice in 0.5 $\times$  SSC and 10 mM DTT at 55°C for 15 min, once in 0.5 $\times$  SSC and 10 mM DTT containing 1 pg/ml 4',6-diamidino-2-phenylindole (DAPI) for 10 min, and then in 0.5 $\times$  SSC and 10 mM DTT for 10 min. Slides were rinsed in water, allowed to air dry, and mounted with 50  $\mu$ l of the antifade reagent ProLong (Invitrogen).

Fluorescent labels were visualized using an epifluorescence microscope (Zeiss Axio Imager A.1) and stimulated by a 100-W high-pressure mercury lamp (HBO100) (Zeiss). Red and blue filters (Zeiss) were used to differentiate between DAPI fluorescence (emission, 461 nm) and rhodamine fluorescence (emission, 603 nm). The software program Axio-Vision, release 4.6.3, was used to record images. By necessity, the length of image exposure differs across tissue sets due to the amount of autofluorescence in different tissues and the magnification used. Exposure and all other microscope settings were kept constant between images of control and *Wolbachia*-containing flies. As a consequence, images can be compared only within the control-test set and not directly across different tissues or across different fly lines. Autofluorescence of fly tissues was observed in the *Wolbachia*-free negative-control flies and differed between tissues types. Fluorescence was attributed to *Wolbachia* presence when surplus to the amount of background fluorescence observed in *Wolbachia*-free negative-control flies. Both punctate and diffuse fluorescence was interpreted as evidence of *Wolbachia*, as punctate staining became diffuse when the focal plane was adjusted throughout tissue sections (data not shown). Each image presented is representative of results observed in multiple sections of at least four individual flies.

**Tissue dissection.** To dissect tissues, male adult 4- to 7-day-old flies were anesthetized using CO<sub>2</sub>, chilled, and beheaded to enable dissection. To assist with tissue isolation and to minimize potential contamination between tissues or by hemolymph, flies were dissected submerged in phosphate-buffered saline (PBS) and visually inspected for purity of tissue. Forceps were used to isolate fly heads and gut (including crop), Malpighian tubules, testes, thoracic ganglia, accessory glands, fat body, or muscle tissue. To increase the amount of starting material, isolated tissues were pooled from five individuals for DNA extraction and immediately snap-frozen in a dry ice and ethanol bath. This was repeated across three separate biological cohorts. DNA was extracted from all tissues on the same day as dissection as described below. *Wolbachia*-free flies were used for negative controls.

**DNA extraction.** To extract DNA from dissected tissues, samples were homogenized in 150  $\mu$ l of chilled PBS for 90 to 180 s, using two 3-mm glass beads (Sigma-Aldrich) and a Mini Beadbeater-96 (Biospec Products). The QIAamp Viral RNA minikit (Qiagen) was used to extract DNA as per the manufacturer's protocol, with the following adjustments. A centrifuge speed of 14,000 rpm was used at each centrifuge step, and samples were eluted and stored in DNase-free water. As

the extraction protocol involves the use of carrier RNA, quantitation of DNA was not performed. Quantitative PCR (qPCR) was used to determine the abundance of the *Wolbachia* surface protein gene (*wsp*) (NC\_0124161.1) relative to the *D. simulans* reference gene, *Actin 5C* (NC\_004354.3) (36).

**Quantitative PCR.** Quantitative PCR was used to determine the abundance of *Wolbachia* in a selection of fly tissues. Platinum SYBR green qPCR SuperMix-UDG (Invitrogen) was used as per the manufacturer's protocol, at halved quantities that resulted in 10- $\mu$ l reaction mixtures that contained 2  $\mu$ l of DNA for relative *Wolbachia* quantitation. Two technical replicates were performed for each sample. If the standard deviation between technical replicate takeoff values was greater than 0.5, the samples were retested. Primer sequences correspond to regions of the *D. simulans* reference gene *Actin 5C* and the *Wolbachia wsp* gene (36). The *Actin 5C* primer sequences used were 5'-GACGAAGAAGTTGCTGCTCTGGTTG-3' and 5'-TGAGGATACCACGCTTGCTCTGC-3', while the *wsp* primer sequences used were 5'-GCATTTGGTTAYAAAATGGACGA-3' and 5'-GGAGTGATAGGCATATCTTCAAT-3' (36). Negative controls included template-free qPCRs and the processing of *Wolbachia*-free flies. Positive controls for the reference gene and *Wolbachia* were included in each qPCR run. The Rotor-Gene 6000 thermal cycler (Corbett Life Sciences, Qiagen) was used with the following profile: 95°C for 2 min, 50°C for 2 min, and 40 cyclic repeats of 95°C for 10 s, 52°C for 10 s, and 72°C for 20 s. This was followed by a standard melt analysis to confirm that only the expected product had been amplified. The abundance of *Wolbachia* relative to the host reference gene was determined using qGENE software. To determine if a correlation existed between *Wolbachia* density and the ability of the strain to mediate protection, a fit model analysis was performed. This was done using the statistical software JMP 8.0.2.2 (SAS), with protection as a fixed variable and strains with protection as a nested variable. A *P* value of <0.01 was interpreted as statistically significant. Graphical representation was performed using GraphPadPrism 5.0a, and standard error is shown.

**Tetracycline treatment to decrease wAu density.** The highly abundant, protective *Wolbachia* strain wAu was selectively decreased in CO flies by treatment with tetracycline. Flies were allowed to lay eggs on normal fly media which contained tetracycline at 0.3  $\mu$ g/ml, 3  $\mu$ g/ml, 9  $\mu$ g/ml, 27  $\mu$ g/ml, 81  $\mu$ g/ml, or 300  $\mu$ g/ml or no tetracycline (21). Hatched larvae fed on the tetracycline-treated media were removed onto fresh tetracycline-free fly medium as 0- to 3-day-old adults. Individual male flies were collected as 4- to 7-day-olds, and DNA was extracted as per the above protocol before qPCR was used to determine *Wolbachia* abundance in individual flies.

**Survival assays.** After the flies were treated with tetracycline to decrease *Wolbachia* organisms to desired densities, they were challenged with virus and survival was monitored. Treatment and viral challenge of *Wolbachia*-free CO flies with 3  $\mu$ g/ml, 27  $\mu$ g/ml, and 300  $\mu$ g/ml or without tetracycline was performed to confirm that antibiotic treatment did not alter survival following viral challenge. Purified DCV isolate EB suspended in Tris (pH 7.4) (16, 25) was maintained at -20°C in aliquots which were used once and then discarded to prevent loss of virus through repeated freeze-thawing. Virus titer was determined by 50% tissue culture infectious dose (TCID<sub>50</sub>) assay (16). Flies were infected by injection as previously described (36). Briefly, 5  $\times$  10<sup>3</sup> infectious units of DCV in 50.6 nl was injected into the hemocoels of 4- to 7-day-old male flies. Negative-control flies were injected with 50.6 nl of PBS. Injections were performed using needles pulled from glass capillaries (Drummond Scientific) and a Nanoject II microinjector (Drummond Scientific). Fly survival was monitored daily, deaths within the first day were discounted as needlestick injury, and the data were normalized accordingly. The challenge assay was replicated using independent fly cohorts. Virus-induced mortality across cohorts is representative where similar *Wolbachia* densities had similar survival outcomes.

**TABLE 1** Summary of *Wolbachia* distribution within fly tissues, across fly-*Wolbachia* combinations that do or do not mediate antiviral protection

Tissue	% of flies in which <i>Wolbachia</i> was detected for indicated fly- <i>Wolbachia</i> combination <sup>a</sup>				
	Protective			Nonprotective	
	CO-wAu	Me29-wMel	DSR-wRi	DSH-wHa	N7NO-wNo
Head	100 (4)	100 (5)	100 (6)	100 (5)	100 (5)
Gut	100 (4)	100 (6)	100 (6)	100 (5)	40 (5)
Malpighian tubules	100 (4)	100 (5)	100 (6)	100 (5)	20 (5)
Thoracic ganglia	100 (4)	100 (4)	100 (5)	100 (5)	100 (5)
Fat body	100 (4)	100 (6)	20 (5)	100 (5)	0 (5)
Muscle	100 (4)	100 (5)	100 (5)	100 (5)	0 (5)

<sup>a</sup> If all individual flies were observed to contain *Wolbachia* within the tissue of interest, a score of 100% was recorded. Where only some individual flies contained *Wolbachia* within the tissue of interest, the percentage of flies that contained *Wolbachia* was recorded. Numbers in parentheses indicate how many individual flies were observed. Percentages do not reflect the *Wolbachia* quantity observed within tissues.

## RESULTS

***Wolbachia* distribution in fly tissues.** Fluorescent *in situ* hybridization was used to qualitatively determine the presence or absence of *Wolbachia* in fly tissues across the five fly-*Wolbachia* pairings. *Wolbachia* was observed in many of the tissues studied, including the head, gut, Malpighian tubules, thoracic ganglion, fat body, and muscle. The *Wolbachia* distribution differed between the fly-*Wolbachia* pairings, and *Wolbachia* was not detected in the tissues of some fly lines (Table 1; see also Fig. S1 to S5 in the supplemental material). In the nonprotective N7NO flies, which have previously been shown to contain the lowest *Wolbachia* density of the flies studied (36), wNO was not identified by fluorescence microscopy in the fat body or muscle of the individual N7NO flies used in this study (Fig. S5). *Wolbachia* was observed in 40% of gut tissue and 20% of Malpighian tubules in the N7NO individuals studied (*n* = 5), indicating that variation in the distribution of detectable *Wolbachia* exists between individuals. Variation between individuals was also observed in DSR flies, in which *Wolbachia* was identified in the fat body of only 20% of the individuals studied (*n* = 5). Despite this, *Wolbachia* was consistently observed in the head and thoracic ganglion of N7NO flies and in the head, gut, thoracic ganglion, Malpighian tubules, and muscle of DSR flies. *Wolbachia* was consistently identified in each targeted tissue of the CO, Me29, and DSH flies in this study but was not homogeneously distributed through different tissue types (Table 1 and Fig. S1 to S3).

Although *Wolbachia* distribution was not ubiquitous across the five fly-*Wolbachia* combinations, the presence or absence of *Wolbachia* from a particular tissue did not correlate with the ability to mediate antiviral protection (Table 1). This was evident as *Wolbachia* distribution was observed within the same tissues in two protective (CO-wAu and Me29-wMel) and one nonprotective (DSH-wHa) fly-*Wolbachia* pairing. In these fly lines, *Wolbachia* was consistently identified in each of the tissues (the head, gut, Malpighian tubules, thoracic ganglion, fat body, and muscle) of each individual in the study (Table 1). This suggests that the presence or absence of *Wolbachia* in fly tissues is not sufficient to mediate antiviral protection.

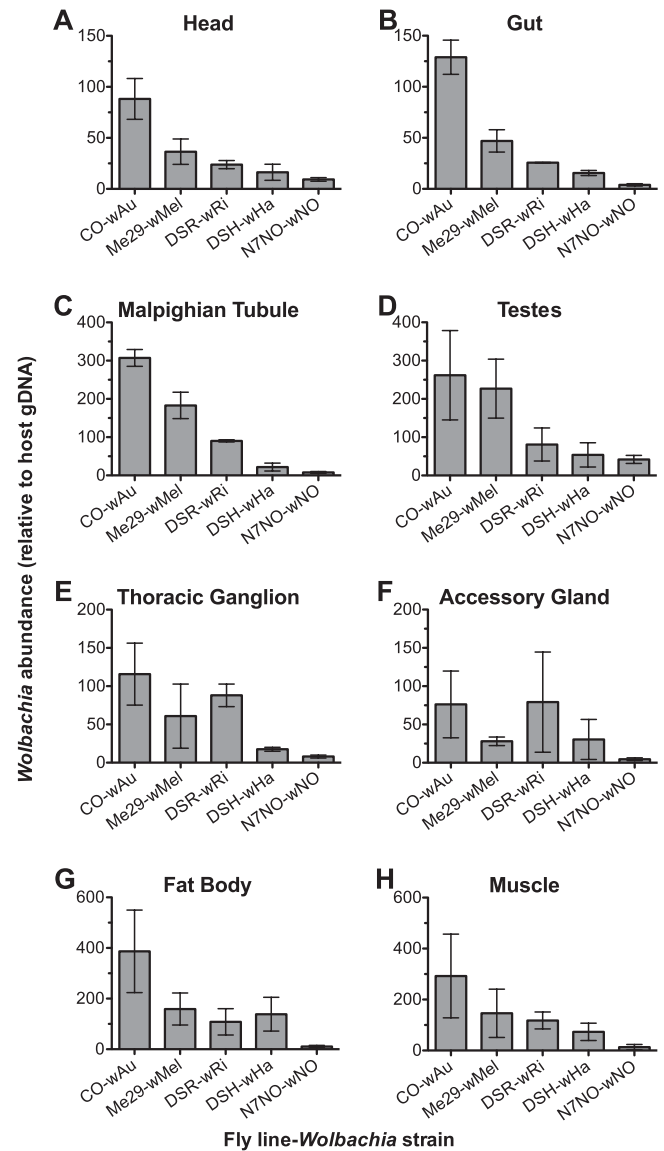
***Wolbachia* density in fly tissues.** Although *Wolbachia* tissue distribution did not correlate with antiviral protection, a differ-

ence in intensity of fluorescence between fly lines was observed. Quantitative PCR was used to determine *Wolbachia* density in the head, gut, Malpighian tubules, testes, thoracic ganglion, accessory glands, fat body, and muscle dissected from protective and non-protective fly lines. A correlation was identified between the *Wolbachia* density and the ability to mediate antiviral protection in three of the tissues studied.

The tissue *Wolbachia* density across the five strains indicated that *Wolbachia* tropism differs between the fly-*Wolbachia* pairings (Fig. 1). For example, in the tissues of CO flies, the greatest mean abundance of *Wolbachia* (*wAu*) was identified in the fat tissue, followed by the Malpighian tubules, muscle, and testes, while lower levels of *wAu* were identified in other tissues. By comparison, DSR contained the most *Wolbachia* organisms (*wRi*) in the testes, and similar amounts of *wRi* organisms were found in the muscle, fat body, Malpighian tubules, thoracic ganglia, and accessory glands, with lower abundances observed in the remaining tissues. The trend of *Wolbachia* abundance that is seen in whole flies (with highest density in CO, then Me29, DSR, and DSH, and the lowest density in N7NO) was reflected in the head, gut, Malpighian tubules, testes, and muscle, but not in the thoracic ganglia, accessory glands, or fat bodies (Fig. 1).

The *Wolbachia* density in tissues across the five fly-*Wolbachia* pairings was correlated with the ability of the strains to mediate antiviral protection. Using a fit model of analysis, with protection as a fixed variable and fly strain with protection as a nested variable, a statistically significant correlation was identified between the *Wolbachia* density and the ability of the strain to mediate antiviral protection in the head ( $P = 0.0052$ ), gut ( $P = 0.0001$ ), and Malpighian tubules ( $P = 0.0001$ ). Of these, the Malpighian tubules appeared to have the largest biological difference between the abundance of *Wolbachia* organisms in strains that do and do not protect, followed by the gut and then the head. Although the testes and the thoracic ganglia ( $P = 0.043$  and  $0.012$ ) could have been interpreted as significant if a less stringent test was used, the *Wolbachia* abundance varied greatly in each of these tissues. High biological variations were also observed in the tissues of some, but not all, fly lines. For example, there was high variation in the absolute *Wolbachia* abundance within the testes of CO flies but not within the testes of N7NO flies. When flies are challenged with DCV, the resulting response to viral challenge is very robust, with little variation between individuals. This suggests that tissues which exhibit high variability of *Wolbachia* tropism are not likely to be relevant in *Wolbachia*-mediated antiviral protection. Likewise, high levels of variation in *Wolbachia* abundance were seen in tissues that did not correlate with the ability to mediate antiviral protection (Fig. 1). The data from this study suggest that *Wolbachia* abundance within the head, gut, and Malpighian tubules may be relevant for antiviral protection.

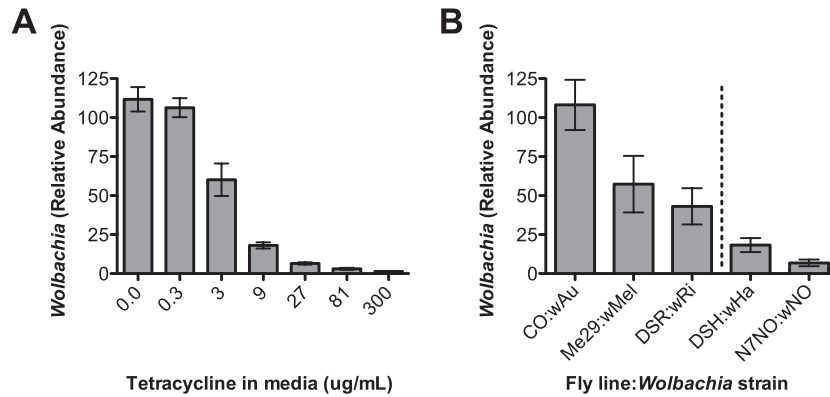
**Reduction of *wAu* density in CO flies leads to loss of antiviral protection.** When CO flies containing the protective *wAu* strain were treated with increasing doses of tetracycline, the *Wolbachia* density in individual flies was decreased in a dose-dependent manner (nonlinear regression,  $R^2 = 0.8525$ , correlation,  $P = 0.0014$ ) (Fig. 2A). Results were consistent between biological replicates of three fly cohorts, and little variation was observed between individual flies. This indicated that *Wolbachia* could be reproducibly decreased in individual flies. The unaltered density of *Wolbachia* was also determined in all of the protective (CO-*wAu*, Me29-*wMel*, DSR-*wRi*) and nonprotective (DSH-*wHa* and



**FIG 1** Relative *Wolbachia* abundance in tissues isolated from *D. simulans* flies. The graphs show the *Wolbachia* abundance relative to a host reference gene (*Actin 5C*) in dissected tissues as determined by qPCR. Tissues are the head (A), gut (B), Malpighian tubules (C), testes (D), thoracic ganglion (E), accessory glands (F), fat body (G), and muscle (H). Dissected tissues were pooled from five individual flies, and the experiment was repeated across three independent fly cohorts. The error bars represent standard errors.  $P$  values were interpreted as significant when  $<0.01$ . A correlation between the *Wolbachia* density within a tissue and the mediation of antiviral protection was observed with the head ( $P = 0.0052$ ), gut ( $P = 0.0001$ ), and Malpighian tubules ( $P = 0.0001$ ) but not in the testes ( $P = 0.0430$ ), thoracic ganglia ( $P = 0.0117$ ), accessory glands ( $P = 0.2279$ ), fat body ( $P = 0.1005$ ), or muscle ( $P = 0.1055$ ).

N7NO-*wNo*) fly-*Wolbachia* combinations using qPCR. *Wolbachia* was most abundant in CO, followed by Me29, DSR, DSH, and then N7NO (Fig. 2B).

To determine if the abundance of *Wolbachia* in whole flies was an important factor in antiviral protection, CO flies containing altered levels of the protective *wAu* were challenged with mortality-inducing DCV (Fig. 3). *Wolbachia*-free CO flies were used as *Wolbachia*-negative controls and injected with DCV or PBS. Flies



**FIG 2** *Wolbachia* abundance in whole flies. Relative *Wolbachia* abundance as determined by qPCR is shown for a high-density, protective *Wolbachia* strain (*wAu*) following treatment with different concentrations of tetracycline (A). Treatment with tetracycline was used to decrease *Wolbachia* abundance in flies and is compiled from three fly cohorts ( $n = 10$  to  $12$ ). Error bars show standard errors. Tetracycline treatment decreases *Wolbachia* abundance in a concentration-dependent manner (nonlinear regression,  $R^2 = 0.8525$ ; correlation,  $P = 0.0014$ ). (B) *Wolbachia* in three protective (CO-*wAu*, Me29-*wMel*, and DSR-*wRi*) and two nonprotective (DSH-*wHa* and N7NO-*wNo*) fly line-*Wolbachia* strain pairings. Male 4- to 7-day-old flies were pooled in groups of five and repeated across three individual cohorts. The density of *Wolbachia* within fly lines correlates with the ability to mediate antiviral protection ( $P = 0.0005$ ). Error bars show standard errors.

that contained *Wolbachia* were treated with tetracycline to obtain mean densities of *wAu* that were equivalent to 3, 5, 6, 30, 39, and 48 copies of *wsp* relative to the host reference gene, *Actin 5C*. *Wolbachia*-containing control flies that were not treated with tetracycline had a *Wolbachia* density that was equivalent to 118 copies of *wsp* relative to host *Actin 5C*. *Wolbachia*-free positive-DCV control flies injected with virus reached 100% mortality 7 days following DCV challenge, which was similar across all biological replicates. *Wolbachia*-containing flies not treated with tetracycline had less than 40% mortality across the duration of the experiment, similar to the virus-free PBS-injected control flies, and similar survival rates were observed with CO-*wAu* flies that contained 48 and 39 relative units of *Wolbachia*. A moderate delay in virus-induced mortality was observed in CO-*wAu* flies that contained 30 relative units of *Wolbachia*. CO-*wAu* flies with 3 to 6 relative units of *Wolbachia* were not protected against viral challenge, as virus-induced mortality was similar to that of *Wolbachia*-free CO flies. Thus, when the abundance of the protective *Wolbachia* strain *wAu* was decreased to levels seen in nonprotective fly lines, *Wolbachia*-mediated antiviral protection was lost.

To determine that tetracycline treatment did not alter viral susceptibility, *Wolbachia*-free flies treated with 300  $\mu\text{g/ml}$ , 27  $\mu\text{g/ml}$ , 3  $\mu\text{g/ml}$ , or no tetracycline were challenged with DCV. Small differences in mortality were observed between *Wolbachia*-free DCV-injected flies in different treatments. As the onset of mortality did not reflect the concentration of tetracycline doses, and the magnitude of the differences was small and not repeatable across fly cohorts, we concluded that tetracycline treatment does not alter the survival of flies under the conditions of the viral challenge assay. The survival data show that the ability of *Wolbachia* to mediate antiviral protection was lost when the level of the protective *Wolbachia* strain *wAu* was decreased (Fig. 3).

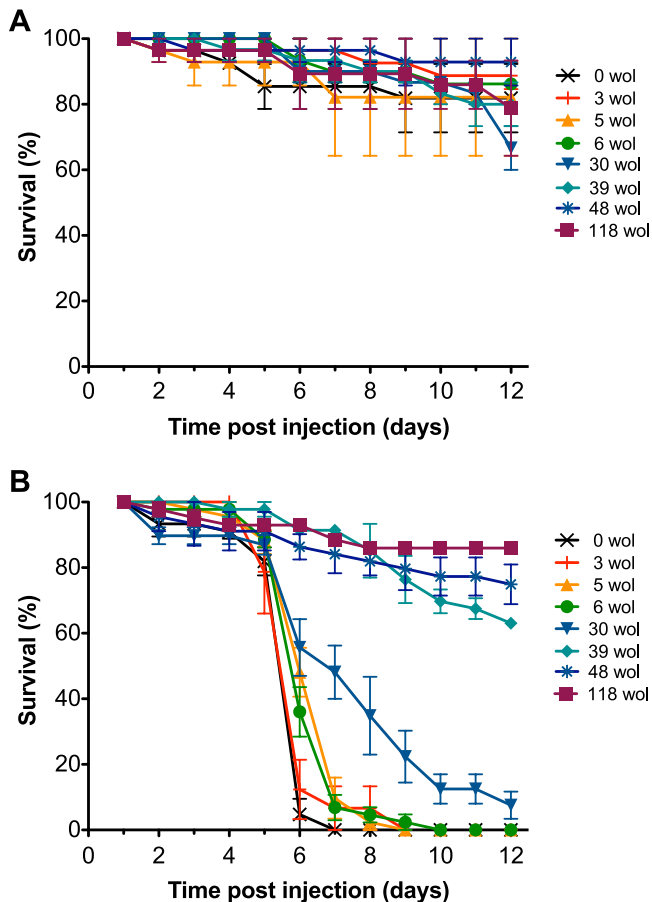
## DISCUSSION

*Wolbachia*-mediated antiviral protection in insects is an intriguing phenomenon, the mechanism of which is not yet understood. The data from this study show that reducing the density of a protective *Wolbachia* strain results in the loss of *Wolbachia*-mediated

antiviral protection. The *Wolbachia* strain *wAu* normally occurs at a high density within the host insect and protects flies against virus-induced mortality (36). When the density of *wAu* in individual CO flies is reduced by antibiotic treatment, the degree of *Wolbachia*-mediated antiviral protection is also reduced or lost completely. This suggests that the *Wolbachia* density in the host is an important factor in the mediation of antiviral protection.

Using five fly-*Wolbachia* combinations, it has previously been shown that there is a correlation between *Wolbachia* density in whole flies and antiviral protection (36). However, the five *Wolbachia* strains are genetically diverse and associated with different *D. simulans* hosts. Thus, in this study, the density of the abundant *wAu* strain was decreased to achieve abundances similar to those of other protective or nonprotective *Wolbachia* strains (36). *Wolbachia* abundance in host insects varies with host-*Wolbachia* combination, larval crowding conditions, environmental conditions, and the age of the insect host (3, 6, 8, 9, 18, 46). Determining *Wolbachia* density under the same conditions as standard viral challenge assays confirms that observations of density correspond with levels of antiviral protection. Interestingly, when the abundance of *wAu* organisms was decreased to an abundance similar to that of the protective *wMel* in Me29 and *wRi* in DSR, *wAu* continued to mediate antiviral protection. When *wAu* was decreased to an abundance that was similar to those of the nonprotective DSH-*wHa* and N7NO-*wNo* combinations, the mortality rate of flies was similar to that of *Wolbachia*-free CO flies. Although the influence of other factors, including phylogenetic differences in strains, remains to be determined, the data from this study suggest that the density of organisms of a *Wolbachia* strain may be important in the ability of the strain to mediate antiviral protection.

Although more work is needed to determine how *Wolbachia* induces the mechanism of antiviral protection, the data from this paper and from the existing literature suggest that a density-related mechanism of antiviral protection is involved (10, 34, 36). Frentiu et al. (10) have shown increased dengue virus inhibition in cell lines that contain greater *Wolbachia* abundances than in those with lower *Wolbachia* abundances, which suggests that *Wolbachia* density is relevant to viral infection in cell culture. In the whole



**FIG 3** Survival of CO flies containing altered abundances of *wAu* following viral challenge. The abundant, protective *Wolbachia* strain *wAu* was decreased in individual CO flies by antibiotic treatment, and then 4- to 7-day-old flies were injected with PBS (negative control) (A) or DCV (B). The graphs show the survival of CO flies which have been treated with tetracycline to decrease the protective *Wolbachia* strain *wAu* to densities equivalent to 3, 5, 6, 30, 39, and 48 copies of *Wolbachia* per host genome (wol) or mock treated (118 wol). Survival of flies is shown from 3 replicates of 15 flies for DCV-injected flies or 2 replicates of 15 flies for PBS controls. CO flies that had previously been cleared of *Wolbachia* (0 wol) were included as *Wolbachia*-negative controls. Data shown are representative of independent survival assays, where comparable densities of *wAu* resulted in similar survival outcomes.

organism, there is likely to be greater variation in both the presence and absence of *Wolbachia* within cell types and the number of *Wolbachia* organisms within particular cells. Despite this variation, the data indicate that the *Wolbachia* density is still important for antiviral protection at a whole-organism level. Moreira et al. (34) have shown in mosquitoes that when *Wolbachia* exhibits a tropism for a particular cell type such as cells in the fat body, dengue virus is limited in the *Wolbachia*-dense tissue. In addition, there is a correlation between *Wolbachia* density across five *D. simulans* lines and antiviral protection (36). Taken together with the data from the current study, this suggests that the density of *Wolbachia* in whole flies is important, possibly as it reflects the *Wolbachia* density within different host tissue types.

The *Wolbachia* distribution of across a variety of somatic tissues in this study shows that the *Wolbachia* strains used here are capable of infecting a wide variety of specialized tissue types. Other studies have identified *Wolbachia* within either numerous

or limited somatic tissues and have reported that tissue distribution varies between the host-*Wolbachia* combinations studied, and that *Wolbachia* distribution and density can change when the bacterium is introduced into a novel host (2, 4, 5, 8, 28, 30). As in this study, Dobson et al. (8) have investigated the qualitative distribution of *wRi* in DSR and identified *Wolbachia* in the head, midgut, Malpighian tubules, ovaries, testes, and muscle using Western blotting against the Wsp protein. Those findings agreed with the data from this study, which identified *Wolbachia* distribution using *Wolbachia*-specific oligonucleotides. As this study identified *Wolbachia* in the same tissues in individuals of two protective (CO and Me29) and one nonprotective (DSH) fly line, the simple presence or absence of detectable *Wolbachia* within the tissues studied is not sufficient to mediate antiviral protection. Interestingly, the protective *Wolbachia* strain *wRi* was only identified in the fat body of one individual ( $n = 5$ ) and at a density similar to that in nonprotective pairing DSH-*wHa*. This indicates that the presence of *Wolbachia* in the fat body is unlikely to be required for the mediation of antiviral protection. In mosquitoes, Moreira et al. (34) found that high levels of *wMelPop*-CLA in the fat body greatly reduced dengue virus within this tissue. However, *wMelPop* and *wMelPop*-CLA occur at a high abundance within many tissues in comparison to other strains, and it is not known if the *Wolbachia* abundance in this or other tissues is correlated with protection (33, 34).

As *Wolbachia* density in whole flies is important for antiviral protection, this study investigated the *Wolbachia* density within tissues dissected from the five protective or nonprotective host-*Wolbachia* combinations. Using qPCR, low *Wolbachia* levels were detected in tissues where *Wolbachia* was not observed using the FISH technique. This is most likely due to differences in sensitivity of the assays and the use of pooled rather than individual samples. The *Wolbachia* density in the head, gut, and Malpighian tubules correlated with the ability of the strains to mediate antiviral protection, suggesting that the *Wolbachia* density at these sites may play a tissue specific role in *Wolbachia*-mediated antiviral protection. Although tissue tropism varied between fly-*Wolbachia* pairings, it was interesting to note that these three tissues did not contain the highest relative *Wolbachia* abundances within individual flies. In fact, across each of the fly lines, the relative *Wolbachia* abundance observed in the head and gut was less than the relative abundance observed in several other tissues.

Specific tissues may have functions that are involved in the response of the host to viral challenge which are altered by high *Wolbachia* densities. The gut forms part of the epithelial barrier and therefore is one of the main sites of microbe entry and initial infection. The Malpighian tubules are involved in the regulation of ions and the excretion of waste, much like the mammalian kidney (reviewed in reference 1). Malpighian tubules have been shown to upregulate the production of nitric oxide synthase and potentially regulate the immune response (reviewed in reference 1). It seems plausible that an abundance of *Wolbachia* organisms in these tissues may alter the host response so that flies with *Wolbachia* are better able to defend against or survive viral challenge. Furthermore, although an altered response could induce a more effective antiviral response, potentially decreasing the production of self-damaging host responses could also result in an overall improved pathology outcome for the host. Further research is needed to determine what role these tissues may play in the *Wolbachia*-host-virus interaction or following viral challenge.

*Wolbachia* is emerging as a useful biocontrol agent to decrease transmission of insect-borne viruses such as dengue virus (7, 13, 14, 19, 23, 34, 38, 42, 43). A better understanding of *Wolbachia* characteristics required for antiviral protection could be useful for predicting *Wolbachia* strains that are likely to mediate strong antiviral protection, either in mosquitoes or in other pathogen-carrying insect species of medical or agricultural significance. The data from this study provide strong evidence that *Wolbachia* density is an important factor required for antiviral protection, although further characterization using different host-*Wolbachia* combinations, *Wolbachia* genetic backgrounds, and viral challenge systems should also be investigated. This study has also shown that *Wolbachia* density within specific tissues (the head, gut, and Malpighian tubules) correlates with the ability to mediate antiviral protection across five diverse *Wolbachia* strains within *D. simulans*.

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