

Wolbachia wAlbB inhibits bluetongue and epizootic hemorrhagic fever viruses in *Culicoides* midge cells

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Abstract

Culicoides midges are hematophagous insects that transmit arboviruses of veterinary importance. These viruses include bluetongue virus (BTV) and epizootic hemorrhagic fever virus (EHDV). The endosymbiont *Wolbachia pipientis* Hertig spreads rapidly through insect host populations and has been demonstrated to inhibit viral pathogen transmission in multiple mosquito vectors. Here, we have demonstrated a replication inhibitory effect on BTV and EHDV in a *Wolbachia* (wAlbB strain)-infected *Culicoides sonorensis* Wirth and Jones W8 cell line. Viral replication was significantly reduced by day 5 for BTV and by day 2 for EHDV as detected by real-time polymerase chain reaction (RT-qPCR) of the non-structural NS3 gene of both viruses. Evaluation of innate cellular immune responses as a cause of the inhibitory effect showed responses associated with BTV but not with EHDV infection. *Wolbachia* density also did not play a role in the observed pathogen inhibitory effects, and an alternative hypothesis is suggested. Applications of *Wolbachia*-mediated pathogen interference to impact disease transmission by *Culicoides* midges are discussed.

KEYWORDS

biting midges, *Culicoides*, orbivirus, population replacement, virus inhibition, wAlbB, *Wolbachia*

INTRODUCTION

Culicoides (Diptera: Ceratopogonidae) midges are small hematophagous insects that are important vectors of veterinary and medically significant viruses (Mills, Michel, et al., 2017). Viruses of particular veterinary importance in the United States include the hemorrhagic orbiviruses: bluetongue virus and epizootic hemorrhagic disease virus (Mills, Michel, et al., 2017; Pfannenstiel et al., 2015). Multiple outbreaks with different serotypes, topotypes (regional variants of particular serotypes) and strains of BTV have been recorded in the United States and Europe in recent decades (De Clercq et al., 2009; Vandenbussche et al., 2009; Velthuis et al., 2010). Although only five serotypes are currently recognized as endemic, 11 additional BTV serotypes have been isolated in the United States since 1999 (Ostlund, 2015). The multitude of serotypes in the United States, with unknown virulence, and as yet unspecified *Culicoides* species transmission vectors, is concerning and

poses a constant threat of disease to US livestock and wildlife (McVey & MacLachlan, 2015; Ruder et al., 2015). The closely related orbivirus, EHDV, causes severe disease in captive and wild cervids, especially white-tailed deer, and typically a subclinical or mild disease in cattle (Becker et al., 2020). Outbreaks result in economic losses to livestock owners due to mortality, trade restrictions and production loss, particularly for the captive cervid industry.

As a result of climate change, the habitat and distribution of many *Culicoides* species have expanded further into northern latitudes (Elbers et al., 2015; Jewiss-Gaines et al., 2017; Mayo et al., 2014; Purse et al., 2005). Changes in climatic conditions have also been shown to affect viral replication resulting in an apparent increase in the vectorial capacity of populations not previously demonstrated to harbour the virus (Guis et al., 2012; Mayo et al., 2014; Purse et al., 2005). Climate change models predict a continuing trend of warmer and wetter weather leading to increased midge population densities and geographic expansion and,

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therefore, expansion of midge-transmitted viruses into new areas in the United States and Europe (Guis et al., 2012; White et al., 2017).

Due to limited vaccine availability, vector control is the best strategy to control midge-transmitted animal diseases. Yet, there are limited effective control methods for biting midges (Benelli et al., 2017; Harrup et al., 2016; Pfannenstiel et al., 2015). With few insecticide products available or registered for use for *Culicoides*, there is a need for novel approaches in *Culicoides* population control to reduce orbivirus transmission. A promising alternative shown in other vector species is the use of *Wolbachia pipientis*, an obligate intracellular maternally inherited bacterium commonly found in invertebrate host species (Werren et al., 2008). In insects, *Wolbachia* bacteria cause alterations in host reproduction known as cytoplasmic incompatibility (CI), which confers a reproductive advantage to infected females (Fallon, 2021; Wang et al., 2021). *Wolbachia* variants used in *Ae. aegypti* have been shown to partially block transmission of Dengue virus (DENV), chikungunya virus (CHIKV), Zika virus (ZIKV) and yellow fever virus (YFV) (Caragata et al., 2016; Flores & O'Neill, 2018; Moreira et al., 2009; Tan et al., 2017; van den Hurk et al., 2012). *Wolbachia* infections in the brown planthopper *Nilaparvata lugens* (Stål) have also been shown to limit ragged stunt virus replication, a virus in the family Reoviridae with BTV and EHDV (Gong et al., 2020). Previous experiments using *Drosophila melanogaster* as a model system have also demonstrated the wMel *Wolbachia* infections inhibit BTV replication (Shaw et al., 2012). Because *Wolbachia* infection confers a reproductive advantage to infected females (Fallon, 2021; Wang et al., 2021), a virus refractory phenotype can be driven into a natural population along with the spreading *Wolbachia* infection (Yen & Failloux, 2020). Recently, *Wolbachia* endosymbionts have been identified in laboratory strains of *Culicoides sonorensis* and in natural populations of other *Culicoides* spp. (Covey et al., 2020; Mee et al., 2015; Pages et al., 2017). Previous studies have also demonstrated that *Wolbachia* infection activates innate immune signalling pathways including the: IMD (immune deficiency) and Jak/STAT (Janus kinase/signal transduction and transcription activators), which have been demonstrated to be involved in antiviral defences in mosquitoes (Pan et al., 2012). The IMD pathway results in the expression of the Relish transcription factor, which induces the expression of antimicrobial peptide genes (AMPs) (Tanji et al., 2007). The STAT pathway and expression of the STAT transcriptional factor, which leads to the activation of immune-related proteins, including cytokines and stress response proteins (Myllymaki & Ramet, 2014; Souza-Neto et al., 2009). Similar immune response pathways have been identified in *Culicoides* midges and may be playing a role in virus transmission (Bian et al., 2010; Nayduch et al., 2014a; Rances et al., 2012).

Although *C. sonorensis* is an important agricultural pest and disease vector, little is known about the effects of its *Wolbachia* symbionts on innate immune response nor the potential impacts they may have on orbivirus transmission. Here, we examined the ability of *Wolbachia* to reduce replication of the orbiviruses BTV and EHDV in *Culicoides* midge cells in vitro as detected by reverse transcriptase quantitative PCR (RT-qPCR) and virus isolation. We also examined transcriptional activation of immune responses of *Wolbachia*-infected cells compared to naïve cells. Our results provide new insights into understanding the novel interaction of *Wolbachia*, orbiviruses and

Culicoides midges, and suggest the potential utility for developing *Wolbachia*-based control approaches.

MATERIALS AND METHODS

Cell cultures and viruses

The *Wolbachia*-infected *C. sonorensis* midge cell line W8-w was generated by transfecting CuVa-W8a cells [USDA, ARS, Arthropod-Borne Animal Diseases Research Unit (ABADRU), Manhattan, KS, USA] with the wAlbB *Wolbachia* strain from *Ae. albopictus* Aa23 cells as previously described (Ghosh et al., 2019). The W8 and W8-w cells were cultured in 25 cm² cell culture flasks (Corning, Glendale, AZ, USA) in Schneider's insect media (ThermoFisher, Waltham, MA, USA) (24.5 g/L) supplemented with 0.4 g/L sodium bicarbonate, 0.0585 g/L L-glutamine, 0.006 g/L reduced glutathione, 0.03 g/L L-asparagine, 18 µL of 10 mg/L bovine insulin and 5% heat-inactivated foetal bovine serum (FBS) (Sigma, St. Louis, MO, USA). All midge cells were incubated at 28°C and passaged at a 1:4 ratio of cells: media every 5–7 days (Ghosh et al., 2019). Orbiviruses (ABADRU stock viruses) used for infection studies included a BTV-13 bovine isolate (2.16 × 10⁷ pfu/mL) and an EHDV-2 white-tailed deer isolate (9.05 × 10⁶ pfu/mL), previously passaged in baby hamster kidney cells (ATCC, Manassas, VA) in Eagles MEM with Earle's salts, 2% FBS, 100 U penicillin/streptomycin sulphate (Sigma). Stock viruses and time course samples were titered on Vero MARU cells (VM; Middle America Research Unit, Panama) grown in M-199E media (with Earle's salts and 2% FBS, 100U penicillin/streptomycin sulphate; Sigma) at 37°C with 5% CO₂ by standard plaque assay.

Virus infection assays and RT-qPCR assays to detect viral RNA

To examine the effects of *Wolbachia* on orbivirus infection, two replicate 25 cm² flasks of W8 and W8-w cells at 80%–90% confluency were inoculated with either BTV-13 or EHDV-2 viruses at MOI 0.25 in 5 mL of media. At 0, 0.5, 1, 2, 2.5, 3, 4, 5, 6 and 7 days post infection (DPI), duplicate flasks were frozen and held at –80°C. 'Time zero' flasks were frozen within 15 min of the inoculum being added. Flasks were thawed and cells were pelleted by centrifuging at 1683 × g for 5 min in 15 mL centrifuge tubes. Pellets were re-suspended in 400 µL of 1X phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄), and 200 µL was used for DNA extractions for *Wolbachia* density determination and the remaining 200 µL for RNA isolations to determine virus quantification and transcriptional activation of immune gene response. To quantify virus copy number, RNA was isolated using RNeasy mini kits (Qiagen, Hilden, Germany), and approximately 1 µg of total RNA was converted to cDNA using a Luna Script Reverse Transcriptase Super Mix (New England Biolabs, Ipswich, MA, USA) following manufacturer's instructions. A fragment of the non-structural NS3 gene for each virus was amplified using cDNA and

primers specific to BTV and EHDV (Table S1). All amplifications were performed as two technical replicates using Platinum 10X SYBR Green qPCR SuperMix-UDG (ThermoFisher) on a Bio-Rad CFX96 qPCR system (Bio-Rad, Hercules, CA USA). The copy numbers of both BTV-13 and EHDV-2 were determined using a standard curve generated from each NS3 gene by analysing 10^1 – 10^8 copies/reaction of two biological and two technical replicates.

Wolbachia infection density determined via RT-qPCR and fluorescent in situ hybridization

DNA was isolated using DNeasy kits (Qiagen) following manufacturer's instructions for cell culture. To determine the density of *Wolbachia* infections in W8-w cells, isolated DNA samples were subjected to qPCR. *Wolbachia* density was determined by amplifying a fragment of the *Wolbachia* *wsp* gene (Table S1) (Sheehan et al., 2016) using Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fisher) on a Bio-Rad CFX96 qPCR system (Bio-Rad) and completed in duplicate. The relative abundance of *Wolbachia* was normalized to the single copy elongation factor 1b gene (Table S1).

Fluorescent in situ hybridization (FISH) was performed on the W8-w and W8 cell lines to confirm the presence or absence of *Wolbachia*, respectively. Cells were grown to 90% confluency at 28°C, the media removed, and the cells trypsinized and resuspended in 5 mL of Schneider's insect media supplemented with 10% FBS. A 300 μ L sample of the cell suspension was added to each well of an eight-well Nunc Lab-Tek Chamber slide (Thermo Fisher). Cells were incubated in the chambered wells for 12 h at 28°C, fixed with 200 μ L 4% formaldehyde (in PBS) for 40 min at room temperature (RT) and washed twice with 300 μ L PBS-Tween (0.1%). Cells were incubated with pre-hybridization buffer [50% deionized formamide, 20% 20 \times sodium saline citrate (SSC) solution, 1% 50 \times Denhardt's Reagent, 10% 1 mol dithiothreitol (DTT), 0.25 mg/mL t-RNA and 0.25 mg/mL poly(A)] for 2 h at RT. Cells were then incubated overnight at 37°C in a moist environment with gentle shaking at 100 rpm (MaxQ 4450 orbital shaker; Thermo Fisher) in hybridization buffer consisting of pre-hybridization buffer supplemented with 200 mg/mL dextran sulfate, 250 mg/litre salmon sperm DNA and fluorescein (FAM)-labelled *Wolbachia* specific probes (Integrated DNA Technologies, Coralville, IA) (Table S1) (Rasgon et al., 2006). After hybridization, cells were washed twice at RT with wash buffer 1 (1 \times SSC augmented with 10 mmol/litre DTT) followed by two washes at 55°C with wash buffer 2 (0.5 \times SSC augmented with 10 mmol/litre DTT), each with gentle shaking as above. Following the wash steps, cells were stained with DAPI at RT for 5 min followed by three 5 min washes with 1X PBS. The cells were then observed using Leica DM IL LED confocal microscope (Leica, Wetzlar, Germany) at 60 \times magnification and photographed with a Leica DFC3000 G camera (Leica) using Leica Acquire software (Leica). All images were processed using ImageJ and Adobe Photoshop (Adobe Systems, Inc, San Jose, CA, USA).

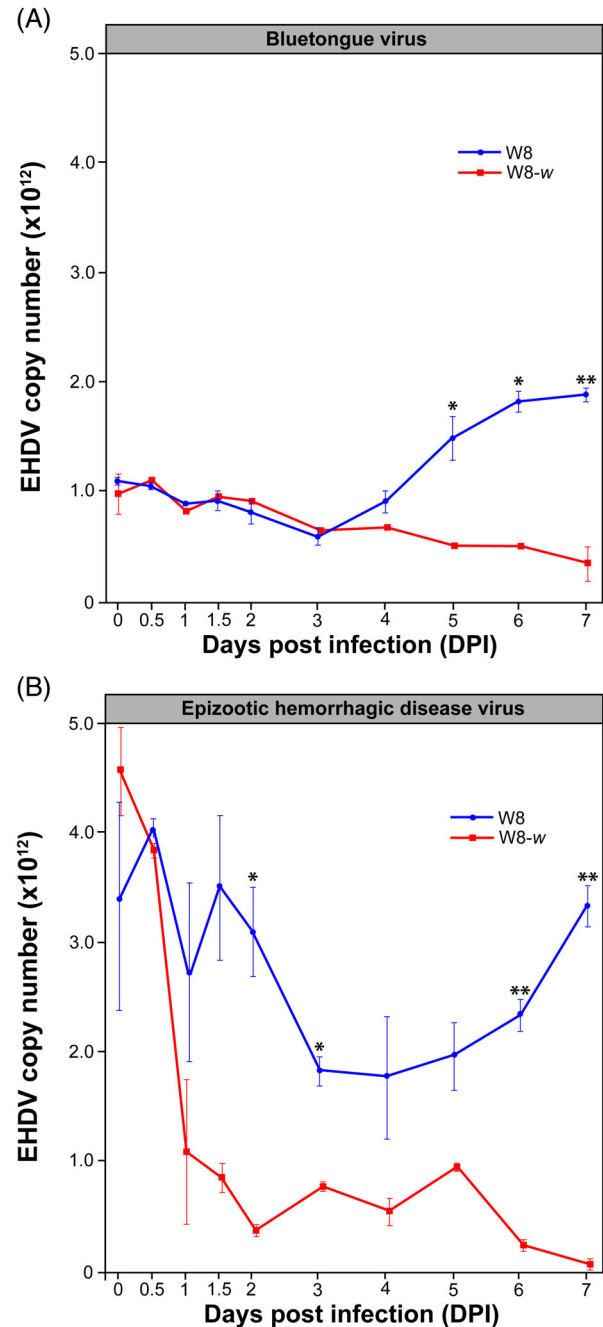


FIGURE 1 Quantitative orbiviral replication as detected by real-time polymerase chain reaction (RT-qPCR) in *Culicoides* cells with and without *Wolbachia* infection. *Culicoides* W8 cell line with *Wolbachia* infection (W8-w; blue) and without (W8; red) from 0 to 7 days post infection (DPI) with (a) bluetongue virus (BTV) and (b) epizootic hemorrhagic fever virus (EHDV). Bars represent means \pm standard error (SEM) calculated across two independent replicate experiments (* $p < 0.05$, ** $p < 0.01$)

Plaque assays to determine virus titers

Based on the RT-qPCR results, a follow-up time course infection study was conducted to determine the effects of *Wolbachia* on infectious orbivirus titers. Duplicate 25 cm² flasks of W8 and W8-w cells

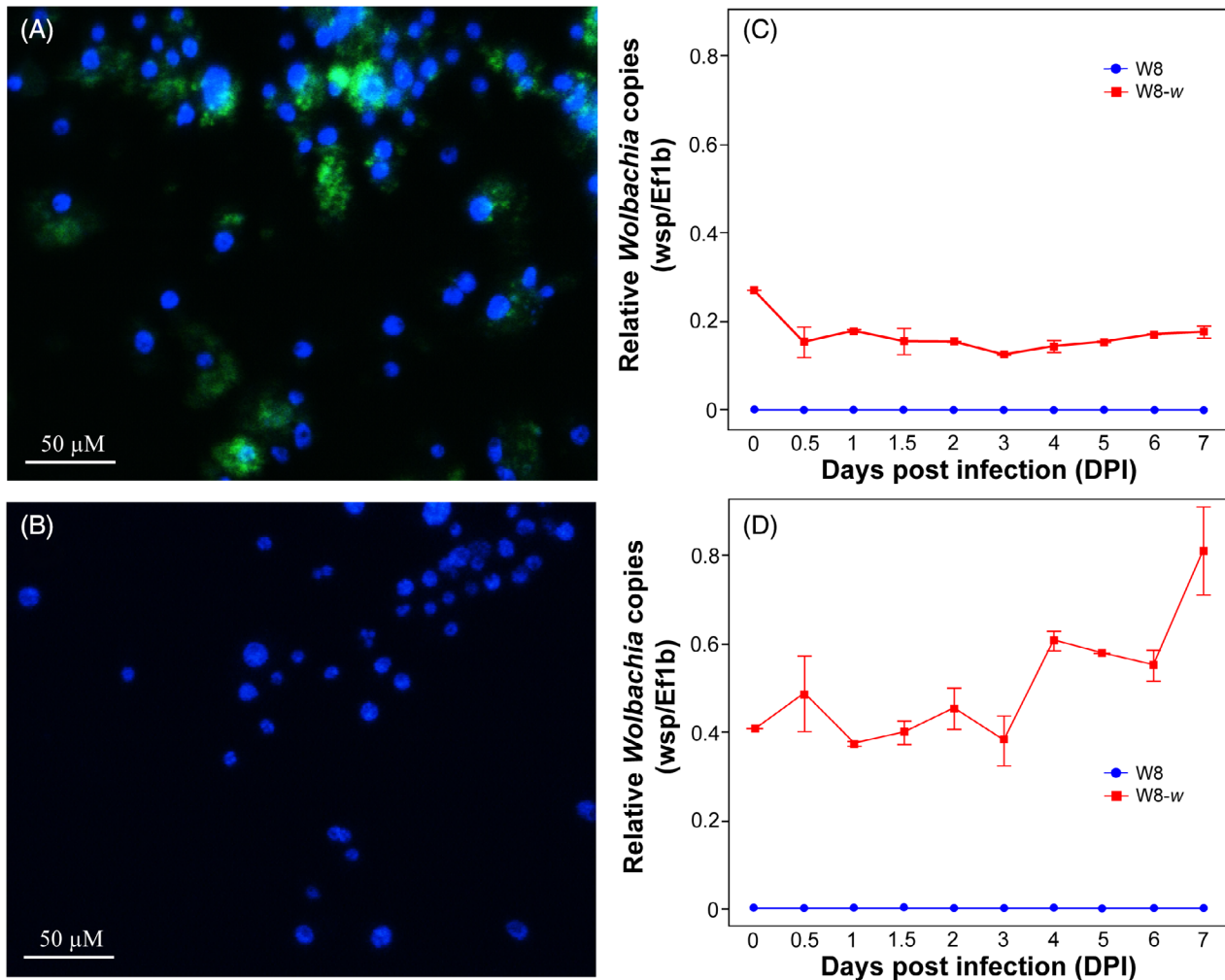


FIGURE 2 (a) Presence of *Wolbachia* infections in W8-w and (b) absence of *Wolbachia* in W8 cells using fluorescent in situ hybridization. Cell nuclei are blue and *Wolbachia* specific probes are shown in green. All images were taken at 60X magnification. (c) *Wolbachia* density in W8-w and W8 cells determined by polymerase chain reaction (qPCR) at different time points post infection with bluetongue virus (BTV) and (d) epizootic hemorrhagic fever virus (EHDV). All *Wolbachia* density measurements when comparing W8-w and W8 cells at each time point for each virus infection type were significantly different ($p < 0.005$). Data are represented as the mean \pm standard error (SEM)

at 80%–90% confluency were inoculated with either BTV-13 or EHDV-2 viruses at MOI 0.25 in 5 mL of media. As orbivirus infection of *Culicoides* cells is non-cytolytic, and virus remains primarily cell-associated, at 0, 1, 2, 3, 5 and 7 DPI, 4 mL of the media was removed, flasks were frozen and held at -80°C . Flasks were thawed, cell suspensions were transferred to microfuge tubes and cell debris was pelleted by centrifugation at $327 \times g$ at 4°C for 5 min. Cleared supernatant was transferred to new tubes and titered by standard plaque assay on VM cells. Two biological replicates were performed for each time point.

Relative quantification of candidate immune gene expression

To quantify host immune gene expression, we used 3 μ L of previously generated cDNA used to quantify virus proliferation using RT-qPCR. Expression of genes involved in the IMD, Toll and Jak/Stat pathways and anti-microbial peptides previously identified in *C. sonorensis* (Table S1)

(Nayduch et al., 2014a) were amplified using Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fisher) on a Bio-Rad CFX96 qPCR system (Bio-Rad). The expression of genes in the IMD, Toll and Jak/Stat pathways were examined because previous studies suggested upregulation in W8-w cells as well as their association with anti-viral inhibitory phenotypes in other insect systems (Ghosh et al., 2019). All reactions consisted of two biological replicates and three technical replicates. Differential gene expression between W8 and W8-w treatments using mean Ct values of the technical replicates was quantified by normalizing to the house-keeping elongation factor 1b gene using the $2^{-\Delta\Delta\text{ct}}$ method.

Statistical analyses

JMP Pro version 16 statistical software (SAS, Cary, NC, USA) was used for all RT-qPCR statistical analyses and GraphPad version 9.2.0 (Prism, San Diego, CA, USA) was used to graph and analyse infectious virus titers. Statistical differences of virus copy number and titers between W8-w and W8 cells were determined using two-way analysis of variance

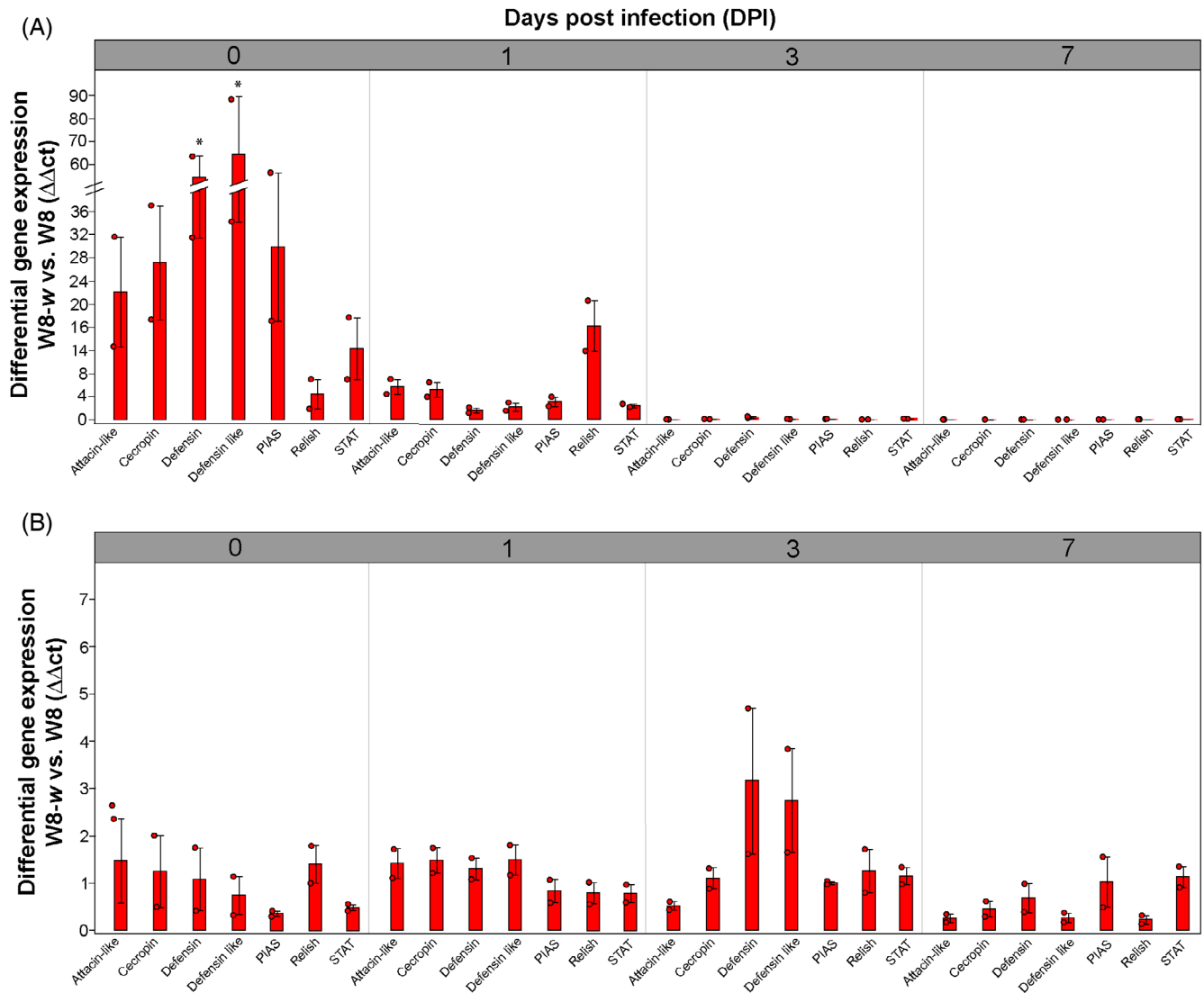


FIGURE 4 Immune gene responses of W8-w and W8 cells when inoculated with (a) bluetongue virus (BTV) and (b) epizootic hemorrhagic fever virus (EHDV). Graphs show the relative expression of target immune genes (x-axis) to house-keeping gene elongation factor 1b at 0, 1, 3 and 7 days post infection. The bars represent mean values from two independent cohorts of cells infected with virus. Statistically significant differences for each gene and separate time point are annotated by an * above each bar ($p \leq 0.05$)

(ANOVA) and Tukey HSD (honesty significant difference) post hoc tests. Statistical differences of *Wolbachia* density between W8-w and W8 cells were determined using Kruskal–Wallis tests and pairwise Bonferroni corrected Wilcoxon tests. Data were checked for normal distribution and equality of variance using a Levene's test. Statistical differences in immune gene expression between W8 and W8-w cell types for each gene were determined using t-tests.

RESULTS

Effects of *Wolbachia* on BTV and EHDV infection

Following experiments that demonstrated *C. sonorensis* W8 cells were permissive to infection with wAlbB (W8-w) (Ghosh et al., 2019), we investigated whether wAlbB infection inhibited BTV or EHDV infection as detected by RT-qPCR targeting the NS3 gene for both viruses. Replication

of BTV was significantly reduced in the W8 cell lines when examining *Wolbachia* infection status (ANOVA, $DF = 1$, $F = 4.38$, $p = 0.04$) and the interaction of DPI and *Wolbachia* infection status (ANOVA, $DF = 9$, $F = 3.03$, $p = 0.01$) (Figure 1a). Replication of EHDV was significantly reduced in the W8 cell lines when examining *Wolbachia* infection status (ANOVA, $DF = 1$, $F = 39.49$, $p < 0.0001$) and the interaction of DPI and *Wolbachia* infection status (ANOVA, $DF = 9$, $F = 5.07$, $p = 0.001$) (Figure 1b).

Wolbachia confirmation and density in response to BTV and EHDV infection

To confirm *Wolbachia* infections in the W8-w cell line, FISH was performed using *Wolbachia*-specific fluorescently labelled oligonucleotides that targeted the *Wolbachia* *wsp* gene (Table S1). Fluorescent microscopy

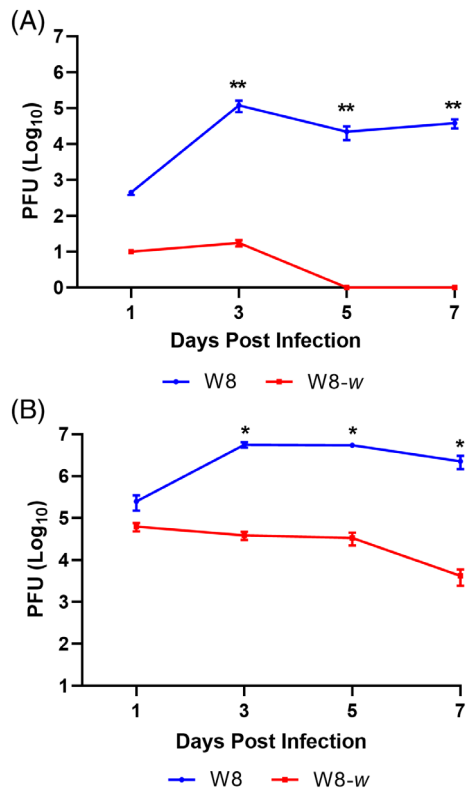


FIGURE 3 Inhibition of orbiviral replication in *Culicoides* cells by *Wolbachia* infection. Detection of infectious virus by plaque assay in W8 (blue) and W8-w (red) *Culicoides* cells infected with (a) bluetongue virus (BTV) and (b) epizootic hemorrhagic fever virus (EHDV). Bars represent means \pm standard deviation calculated across two independent replicate experiments. (* $p \leq 0.05$, ** $p \leq 0.01$)

confirmed the presence of *Wolbachia* in the cytoplasm of the W8-w cell line (Figure 2a). No fluorescent signal for *Wolbachia* was observed in the W8 cell line (Figure 2b). To investigate whether virus proliferation was influenced by *Wolbachia* infection dynamics and/or if *Wolbachia* density was influenced by virus infection, we quantified *Wolbachia* density using qPCR targeting the *wsp* gene from the infection time course samples. W8-w cell lines inoculated with BTV maintained a relative *Wolbachia* density of approximately (0.19 ± 0.02) (mean \pm standard deviation, SD) compared to W8 cells (Kruskal-Wallis, DF = 1, Chi-sq 33.44, $p < 0.001$), suggesting BTV infection did not impact *Wolbachia* replication. However, *Wolbachia* density was observed to increase over time in EHDV inoculated W8-w compared to W8 cells (Kruskal-Wallis, DF = 1, Chi-sq = 27.35, $p < 0.001$) with a relative *Wolbachia* density of (0.41 ± 0.02) (mean \pm SD) at day 0 and (mean \pm SD) (0.81 ± 0.14) at day 7.

Effects of *Wolbachia* on BTV and EHDV infectious titers

Based on the RT-qPCR results, a follow-up study was conducted to quantitate *Wolbachia*-induced reduction of infectious orbivirus

titers in W8 cells using a plaque assay. Significant reduction in infectious BTV titers was observed in W8-w cells by day 3, with complete clearing of the virus by day 5 ($p < 0.05$, t-tests) (Figure 3a). For EHDV, a significant reduction in infectious virus titers was observed in W8-w cells from day 3 through day 7 ($p \leq 0.05$, t-tests) (Figure 3b).

Effects of *Wolbachia* on host cell gene expression in response to BTV and EHDV infection

To determine whether reductions in virus proliferation in *Wolbachia*-infected cells was related to the stimulation or priming of the *Culicoides* innate immune system in vitro, we quantified the expression of a panel of immune genes. The expression levels of seven genes from either the Jak/STAT or IMD pathways or that encode for antimicrobial peptides in the W8-w and W8 cell lines were compared for two independent cohorts of cells inoculated with BTV and EHDV. In BTV-inoculated cells, four genes encoding representatives of the immune antimicrobial effector molecules defensin, defensin-like, attacin-like, and cecropin were significantly upregulated in the presence of *Wolbachia* within 15 min after virus inoculation (time 0) ($p \leq 0.05$, t-tests) (Figure 4a). In addition, differential mRNA expression between W8-w and W8 cells was observed for a subset of the genes (E3 SUMO-protein ligase (PIAS), signal transducer and activator of transcription (STAT) and the transcription factor relish) from the Jak/STAT and immune deficiency (IMD) signalling pathways at time 0 ($p \leq 0.05$, t-tests) (Figure 4a), while little upregulation of these genes was observed at 1, 3 and 7 days post BTV inoculation. In contrast, when quantifying gene expression in EHDV inoculated cells, no significant changes to gene expression were observed at 0, 1, 3 and 7 days post inoculation in W8-w and W8 cells (Figure 4b).

DISCUSSION

As an important veterinary disease transmission vector, there is a critical need to determine whether *Culicoides* cells can support a *Wolbachia* infection, how they interact and the effect *Wolbachia* has on BTV and EHDV productive infections. In the absence of such knowledge, an evidence-based framework for the subsequent development of a *Wolbachia*-based *Culicoides* BTV and EHDV disease control approach will remain unlikely. With this in mind, we examined the mechanism of *Wolbachia*-mediated inhibition of BTV and EHDV infection in *C. sonorensis* W8 cells. Here, we have demonstrated that the wAlbB *Wolbachia* infection, donated from Aa23 cells, significantly reduced replication of these two closely related orbiviruses in *Culicoides* cells. The wAlbB infection was an appropriate candidate for the *Wolbachia* strain to examine for a viral inhibitory phenotype because previous studies demonstrated an upregulation of immune response when wAlbB was introduced into naïve host cells suggesting the potential for a viral inhibitory phenotype (Ghosh et al., 2019). Furthermore wAlbB infections have also been associated with viral

inhibitory phenotypes in other dipterans, particularly mosquitoes (Ant et al., 2018; Bian et al., 2010; Flores et al., 2020).

Wolbachia density has been suggested as a factor that impacts virus replication and mediating inhibitory effects in some insect tissues (Bian et al., 2010). Previous research has shown *Wolbachia* density in *Ae. albopictus* cells and adults is directly related to virus inhibitory effects with higher densities (>100 *Wolbachia*/cell) resulting in more significant pathogen interference (Lu et al., 2012). In contrast, *Wolbachia* density had no inhibitory impact on Flock House virus replication in *Drosophila* hosts, but *Wolbachia* density was instead impacted by the virus infection, suggesting these interactions can affect both arbovirus and the *Wolbachia* symbiont (Kaur et al., 2020). In this study, *Wolbachia* densities were similar to previously reported densities in W8-w cells without BTV infections (0.77 ± 0.26 *Wolbachia*/cell) (Ghosh et al., 2019), suggesting no correlation with pathogen inhibitory effects in *Culicoides* cells infected with BTV. A marginally higher but still low-density *Wolbachia* infection was observed in EHDV infected cells when compared to previously observed *Wolbachia* densities in W8-w cells (Ghosh et al., 2019). A minor increase in *Wolbachia* density after 3 days post-EHDV infection correlated with a decrease in EHDV copy number and reduction of virus in the later time points post infection. Our data suggest *Wolbachia* and the observed EHDV and BTV virus inhibitory effect may not strictly be dependent upon *Wolbachia* density in W8-w *Culicoides* cells.

An alternative mechanism other than *Wolbachia* infection density that may be impacting virus proliferation is an increased immune response primed by *Wolbachia* infection (Moreira et al., 2009; Rances et al., 2012). Our observations support this hypothesis in that BTV virus infection was significantly reduced in RT-qPCR assays and virus was cleared by day 5, in the absence of a corresponding significant increase in *Wolbachia* density, but in the presence of high expression levels of immune genes, particularly immune effectors such as antimicrobial peptides regulated by the Toll and IMD pathways, immediately after virus exposure in W8-w cells. BTV has been shown to enter cells within the first 5 min of infection (Du et al., 2014). As mentioned above, EHDV levels decreased with a slight corresponding increase in *Wolbachia* density, but in the absence of significant immune responses. A similar rapid immune response to BTV-inoculated W8-w cells was not observed in EHDV-inoculated W8-w cells. Similar results were previously reported for *Ae. albopictus* where cells infected with a wMel infection at a higher density than the naturally infected wAlbB and wAlbA infections had little to no gene expression of innate immune pathways nor antioxidant ROS producing functions associated with the antiviral phenotype (Molloy & Sinkins, 2015). In contrast, previous work with *Drosophila simulans* and multiple *Wolbachia* strains found in a similar host genetic background suggests there is a strong correlation between *Wolbachia* density in host tissues and viral inhibitory phenotype (Martinez et al., 2014). Hence, there may be other factors, a combination of various mechanisms, or other immune genes not investigated here that are influencing the ability of *Wolbachia* to impact EHDV and perhaps BTV virus proliferation.

While not tested in this study, previous work has demonstrated that the host cell, virus and *Wolbachia* compete for intracellular host resources, including amino acids, lipids and intracellular space. This competition for intracellular resources can impact both virus proliferation and potentially *Wolbachia* density (Caragata et al., 2013; Caragata et al., 2014). *Wolbachia* bacteria require fatty acids and cholesterol for their cellular metabolism and lack the biosynthetic capability to synthesize cholesterol, which suggests a need to obtain it from the host insect cell (Wu et al., 2004). The role of lipid transport in *Wolbachia*'s capacity to inhibit DENV in vivo and in vitro has been previously demonstrated (Caragata et al., 2013; Koh et al., 2020). Our observed results, and previous studies taken together suggest the potential for a similar competition for intracellular resources could also be contributing to the observed BTV and EHDV inhibitor effect in W8-w infected cells. However, additional testing is required to support this hypothesis.

Overall, the results presented here agree with previous studies that *Wolbachia*-induced pathogen inhibition is complicated, and *Wolbachia* effects on viruses may be specific to the host cell, *Wolbachia* strain and the virus. Furthermore, multiple factors such as *Wolbachia* density, host cell immune response and competition for resources may be contributing to viral inhibitory effects. Demonstration of wAlbB *Wolbachia* infections impacting BTV and EHDV replication in vitro is an initial step towards the potential application of *Wolbachia*-based population and disease control approaches targeting *Culicoides* species. Previous studies have demonstrated low-density natural *Wolbachia* infections in multiple *Culicoides* species worldwide, but to date, there is no evidence of any reproductive phenotypes that could be used to manipulate natural populations or for disease control (Covey et al., 2020; Mee et al., 2015; Pages et al., 2017). Naturally occurring *Wolbachia* infections have also recently been demonstrated to result in virus reductions in *D. melanogaster* (Cogni et al., 2021). With the recent identification of *Wolbachia* infections in wild-type populations of *Culicoides*, it would be interesting to determine if any naturally occurring infections result in a virus inhibitory phenotype. Transfection experiments to introduce novel *Wolbachia* infections into *Culicoides* species is crucial to determine if *Wolbachia* can inhibit BTV or EHDV proliferation in important *Culicoides* vectors such as *C. sonorensis*. Unfortunately, both of the aforementioned potential experiments are complicated by the current inability to colonize many *Culicoides* species. Our results provide the impetus for the next logical step, which is to examine whether similar virus inhibitory effects occur in *Culicoides* in vivo and determine if stable maternally inherited novel *Wolbachia* infection(s) can be established in a representative *Culicoides* species such as *C. sonorensis*.

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AUTHOR CONTRIBUTIONS

Corey L. Brelsfoard, Barbara S. Drolet and Megan L. Matthews conceived and designed the study, Megan L. Matthews, Hunter O. Covey and Barbara S. Drolet collected the data, Corey L. Brelsfoard, Megan L. Matthews and Barbara S. Drolet analysed the data, and Megan L. Matthews wrote the first draft of the manuscript. All authors contributed to the final draft of the manuscript.

DATA AVAILABILITY STATEMENT

The *Culicoides* cell cultures that support the findings of this study are available from the United States Department of Agriculture Agricultural Research Service Arthropod-Borne Animal Diseases Research Unit, through material transfer agreements. Raw data is available from the authors upon reasonable request and with the permission of the U.S. Department of Agriculture, Agricultural Research Service. Orbivirus infection data will be available within 30 months of publication at AgDataCommons at the National Agriculture Library.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Table S1 Primer sequence use to amplify host immune pathway gene expression, fluorescent in situ hybridization (FISH), and to quantify bluetongue virus (BTV), epizootic hemorrhagic fever virus (EHDV), and *Wolbachia* copy number.

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