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Wolbachia-mediated antiviral protection is cell-autonomous

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Abstract

Vector-borne viral diseases pose significant risks to human health. To control the transmission of these viruses, a number of approaches are required. The ability of the intracellular bacteria *Wolbachia* to limit viral accumulation and transmission in some arthropod hosts, highlights its potential as a biocontrol agent. Whilst *Wolbachia* can reduce the transmission of several epidemiologically important viruses, protection is not consistent amongst all insects, viruses and strains of *Wolbachia*, which confounds elucidation of the mechanisms that underly this protection. Evidence of different mechanisms has emerged, but is not always consistent, suggesting the tripartite interaction may be complex. Here we provide evidence that *Wolbachia*-mediated antiviral protection is dependent on the presence of *Wolbachia* in individual cells, and cannot be conferred to surrounding cells. Our results suggest that protection is cell-autonomous, and this has several mechanistic implications, which can direct future research.

Wolbachia pipientis is an endosymbiotic bacterium found naturally in an estimated 50% of insect species [1–3]. The bacterium is well known for its ability to spread through populations via reproductive parasitism [4]. Interestingly, the presence of *Wolbachia* is sometimes correlated with lower viral load and reduced virus-induced pathology in its host insect; a mutualistic relationship, which may increase host fitness. This discovery was first made in the model organism *Drosophila melanogaster*, where *Wolbachia* was shown to confer resistance to several different RNA viruses [5, 6]. In the presence of *Wolbachia,* flies can carry a reduced viral load, which is associated with an increase in host lifespan.

This finding sparked research into the potential of *Wolbachia* as a biocontrol agent, with the capacity to impact vector-borne transmission of diseases. Whilst the most common mosquito vector, *Aedes aegypti*, lacks a natural *Wolbachi*a infection, the *w*MelPop, *w*Mel and *w*AlbB strains have been stably introduced into this vector [7–9], which has led to experimentally decreased levels of several different viruses including Dengue virus (DENV), Chikungunya virus (CHIKV) and Zika (ZIKV) virus [10–15]. Currently, the World Mosquito Program is releasing *Wolbachia*-infected mosquitos in 12 different countries to try and alleviate the burden of mosquito-borne diseases, primarily DENV.

Despite *Wolbachia's* potential to change the way we control virus infection in mosquito vectors, the mechanisms underlying antiviral protection remain to be fully elucidated. The difficulty in identifying mechanisms behind the protection stem not only from the limitations of studying an intracellular symbiont, but also because *Wolbachia* protection is not ubiquitous [16–21]. Whether protection is conferred at all, and to what extent, can be dependent on the host species, the virus, the strain of bacteria, as well as the source of the *Wolbachia* infection (i.e. natural, stable or transiently transinfected) [22–24].

Whilst low *Wolbachia* density has been implicated in some symbioses that lack the protection phenotype [16, 18, 21, 25–27], differential protection suggests that the interaction between *Wolbachia* and host may be complex. Evidence for several mechanisms have been found in different systems, including competition between virus and *Wolbachia* for host resources, immune priming, involvement of reactive oxygen species (ROS) and oxidative homeostasis [28–32], however none of these can fully explain the antiviral protection. One facet of *Wolbachia* infection that is typically consistent amongst protective strains, is higher *Wolbachia* density generally correlates to lower viral load [14, 16–18, 25, 33–35]

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Wolbachia density can vary depending on tissue type, and there is correlative data that suggests this tissue tropism is important in *Drosophila* [35], although tissue-specific correlation was not reported in *Ae. aegypti* [36]. Amuzu and McGraw [36] posited that as protection is not dependent on any tissue type, *Wolbachia*-mediated protection must work in either a systemic way, or through a localized mechanism that is common to diverse tissue types.

Whether protection is cell-autonomous would have implications for the mechanisms of protection. The current literature, using immunofluorescence assays, generally shows a lack of co-localization of *Wolbachia* and virus within the same cell [14, 34]. This lack of correlation of immunofluorescence signal for protective strains raises the possibility that *Wolbachia* presence excludes virus from cells, which suggests a direct interaction may be occurring between the two, rather than a systemic response. The relationship between *Wolbachia* density and viral titre, as well as the presence of *Wolbachia* in somatic as well as germline tissues, suggests that protection works at a cellular level. This is also consistent with evidence that *Wolbachia* interferes early in the infection cycle of the virus [37], blocking it from proliferating and accumulating within a cell. Although low frequencies of co-localization have been reported, this is usually at low densities of *Wolbachia* [25], or in a system where protection has not been demonstrated [38]. Whether *Wolbachia* must be present inside a cell to protect would provide insight into potential mechanisms, but this idea has not been tested experimentally.

All experiments presented utilized the JW18 cell line. JW18 cells are a *Wolbachia*-infected line derived from *Drosophila melanogaster* embryonic tissue [39], and are persistently infected with the *w*Mel strain of *Wolbachia*. These cells were received from the Kohl lab at the University of Glasgow, and a paired *Wolbachia*-cured JW18 line (designated JW18TET) was generated by culturing cells with 10 µg ml^{-1} tetracycline for eight passages. The absence of *Wolbachia* was confirmed using qPCR, after which the cells were passaged in antibiotic-free media for eight passages to allow the cells to recover. All JW18 and derivative cell lines were maintained at 27 °C in Sang and Shield's M3 insect media (Sigma), supplemented with 10% FBS. *Wolbachia* density was frequently checked using qPCR as previously described [17], to ensure high levels of *Wolbachia* were maintained.

Previous studies have found *Wolbachia*-mediated protection in several different *Drosophila* species, including *Drosophila melanogaster*. The presence of *Wolbachia* in the JW18 cell line has been shown to confer resistance against Semliki forest virus and Sindbis virus (SINV) [37, 40]. The first series of experiments were undertaken to confirm that *Wolbachia*-infected JW18 cells were protected when challenged with divergent RNA viruses Drosophila C virus (DCV – *Dicistroviridae*) and SINV (*Togaviridae*). JW18 and JW18-Tet cells were infected with DCV and SINV at an m.o.i of 0.001 and an m.o.i. of 1, respectively. After 72 h of infection, samples were harvested in RiboZol. After RNA extraction (as per the manufacturer's instructions), samples were DNase treated with RQ1 RNase-free DNase (Promega), and SuperScript III Reverse Transcriptase was then used for cDNA synthesis, as per the manufacturer's protocol, using random primers.

cDNA templates were analysed via qPCR, using Platinum SYBR Green qPCR SuperMix-UDG kit (Theromofisher), according to the manufacturer's protocols. Gene-specific forward and reverse primers were used for DCV (DCV F: 5′ AGGCTGTGTTTGCGCGAAG 3′, R: 5′ AATGGCAAGCGC ACACAATTA 3′) and SINV (SINV F: 5′ CACAGTGTACGA CCGTCTGAA 3′, R: 5′ AACGGTTCCGGTCTTGTAGTC 3′), and viral RNA accumulation was measured by comparing the relative abundance of these to the reference gene *Rpl32* (*Drosophila* gene F: 5′ TCCTACCAGCTTCAAGATGAC 3′, R: 5′CACGTTGTGCACCAGGAACT 3′). All reactions were done in duplicate and repeated if the standard deviation between technical replicate take-off values was greater than 0.5. The Rotor-gene thermal cycler (Corbett Life Sciences) was used with the following cycle: 50 °C for 2 min, 95 °C 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. To confirm that all genomic DNA had been removed, an Reverse transcriptase minus control was performed using RNA as a template. Statistical analysis was performed using Welch's *t*-tests in GraphPad Prism 8; this test does not assume equal variances. Three independent biological replicates were conducted with up to three repeats per replicate.

Compared with the *Wolbachia*-free JW18-Tet line, there was a significant reduction in accumulation of both DCV and SINV RNA in the presence of *Wolbachia* (JW18 line). For DCV, viral genomic copies were reduced about tenfold in *Wolbachia*-infected cells (1.3+/-0.1, n=8) compared to the control (12.3+/-0.5, n=8) (Welch's *t*-test, df=7.8, P<0.0001), and for SINV viral titres were reduced by about 12-fold (JW18 0.004+/-0.0002, n=6; JW18-tet 0.051+/-0.003, n=6: Welch's *t*-test, df=5.1, P<0.0001), indicating that the presence of *Wolbachia* was having an inhibitory effect on both viruses (Fig. 1). This finding is parallel to the protection phenotype observed in the whole organism [6, 40].

Once *Wolbachia*-mediated antiviral protection was confirmed in JW18 cells, we performed a transwell assay to determine whether protection is cell-autonomous. The transwells used in this study were small, polyester supports that sit inside of a 12-well plate and have a porous membrane bottom that sits above the bottom of the well, allowing the adherent cells below to be physically separated from the cells on the membrane above. The experimental design is shown in Fig. 2a. Cells were seeded in either untreated 12-well polystyrene-cell culture plates (Costar), or on polyester transwell inserts (Corning) that have a pore size of 0.4 µm. The smallest pore size of 0.4 µm was chosen as it was expected to be small enough to allow virus to pass through, but not *Wolbachia,* based on electron microscopy images of *Wolbachia* size [41, 42]. An initial experiment was performed to assay whether *Wolbachia* could pass through the pore size 0.4 µm or larger 3.0 µm. Cells were left to co-culture for 3 days to allow the potential transfer *Wolbachia* from the JW18 cells in the transwells to the JW18-TET cells below. *Wolbachia* was not detected in

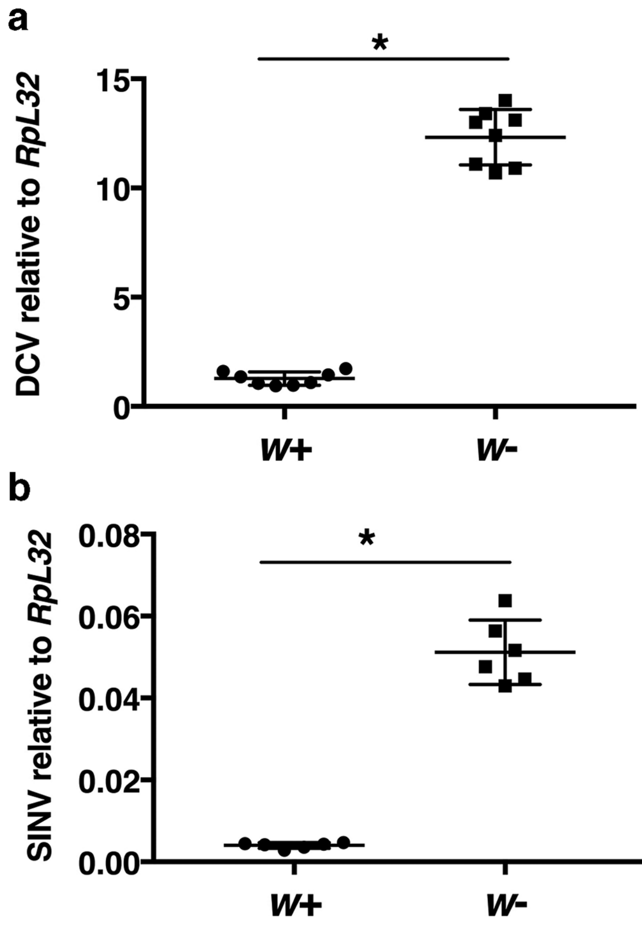

Fig. 1. *Wolbachia*-infected JW18 cells carry a significantly lower viral load compared to *Wolbachia*-free cells. JW18 (*W*+) and JW18 tetracycline treated cells (*W*-) were infected with either DCV (a) or SINV (B) at an m.o.i. of 0.001 or an m.o.i. of 1, respectively. After 72 h of infection, samples were harvested and RT-qPCR was performed to determine the amount of viral RNA accumulation, relative to a *Drosophila* housekeeping gene *Rpl32*. Each dot represents a single-cell culture-assay well (taken across three independent biological replicates), with mean represented by horizontal lines and standard deviation indicated. Welch's *t*-test (A. n=8, df=7.8; B. n=6, df=5.1) was performed and significance is indicated by an asterisk (DCV $P<0.0001$; SINV $P<0.0001$).

the lower cells, suggesting that under the conditions used the *Wolbachia* did not pass through the pore and enter the cells below (Fig. 2b). The transwells therefore allow us to physically separate *Wolbachia*-infected from uninfected cells, while still sharing culture media containing virus and extracellular components. Further experiments were performed in the same manner, but with the addition of virus, to determine if *Wolbachia* presence inside the cultured cells was necessary to confer protection, or if *Wolbachia* could confer protection via signalling or other intercellular means.

The experimental setup is shown in Fig. 2a, with the addition of either DCV or SINV at an m.o.i. of 0.001 or an m.o.i. of 1, respectively. After 48 h of infection, cells in the bottom of the plate (not the transwells) were harvested and viral accumulation was analysed using RT-qPCR. For both DCV and SINV

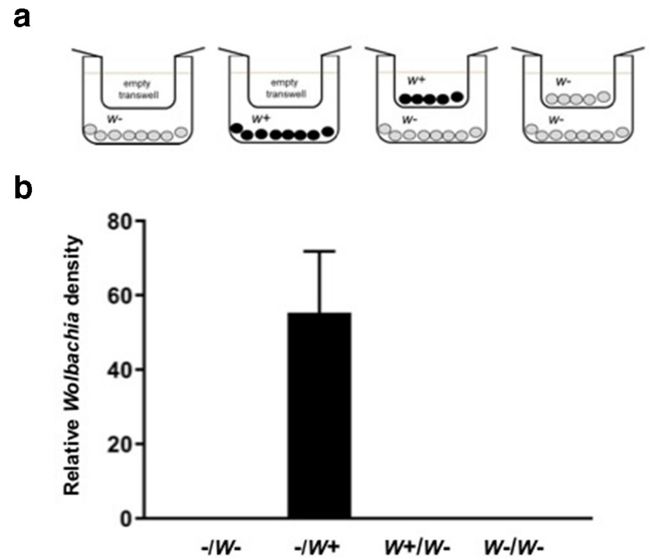

Fig. 2. *Wolbachia* transfer from one culture to another can be prevented using a 0.4 µm transwell insert. (a) Experimental design for transwell experiments. *Wolbachia*-infected (*w*+) and non-infected (*w*-) JW18 cells were co-cultured in transwells separated by a membrane with 0.4 µm pores in four different combinations. (b) Cells were co-cultured in transwells, as per (a), for 3 days before cells in the bottom of the wells were harvested. *Wolbachia* genomic DNA was extracted using the QIAamp Viral RNA extraction minikit (Qiagen), and qPCR was then performed, using the *Wolbachia* primers to *Wsp*, to determine the amount of *Wolbachia* present relative to host housekeeping gene *Rpl32*. For each treatment n=4 and the vertical bar represents standard deviation.

the well containing *Wolbachia*-infected cells at the top of the transwell and *Wolbachia*-uninfected cells at the bottom, had a viral load comparable to the *Wolbachia*-free controls (Welch's *t*-test, n=6 for each treatment, DCV df=9.5, P=0.25 and SINV df=9.9, P=0.83), suggesting that protection was not conferred to the cells below (Fig. 3). This was in contrast to the positive controls where *Wolbachia*-infected cells had significantly reduced viral RNA compared to *Wolbachia*-free cells (Fig. 3, Welch's *t*-test, n=6 for each treatment, DCV df=5.9, $P<0.0002$; SINV df=5, $P<0.0002$). Thus there was no evidence of cell-signalling between physically separated cells, although the experimental design did not test for inter-cell-signalling between adjacent cells. The results are consistent with our hypothesis, that the physical presence of *Wolbachia* inside of a cell is required to confer antiviral protection.

These data are also consistent with other studies, which point towards cell-autonomous protection. It is supported by immunofluorescence data suggesting that *Wolbachia* and virus do not co-localize in cells, as well as observations across many studies in different systems that higher *Wolbachia* density is correlated with higher protection [14, 16, 18, 26, 27, 34, 43, 44]. It is not possible to extrapolate from the current results as to whether the presence of *Wolbachia* excludes the virus from entering the cell completely, or whether once inside the cell, a direct interaction is occurring between *Wolbachia* and virus that leads to its inability to replicate and accumulate in that

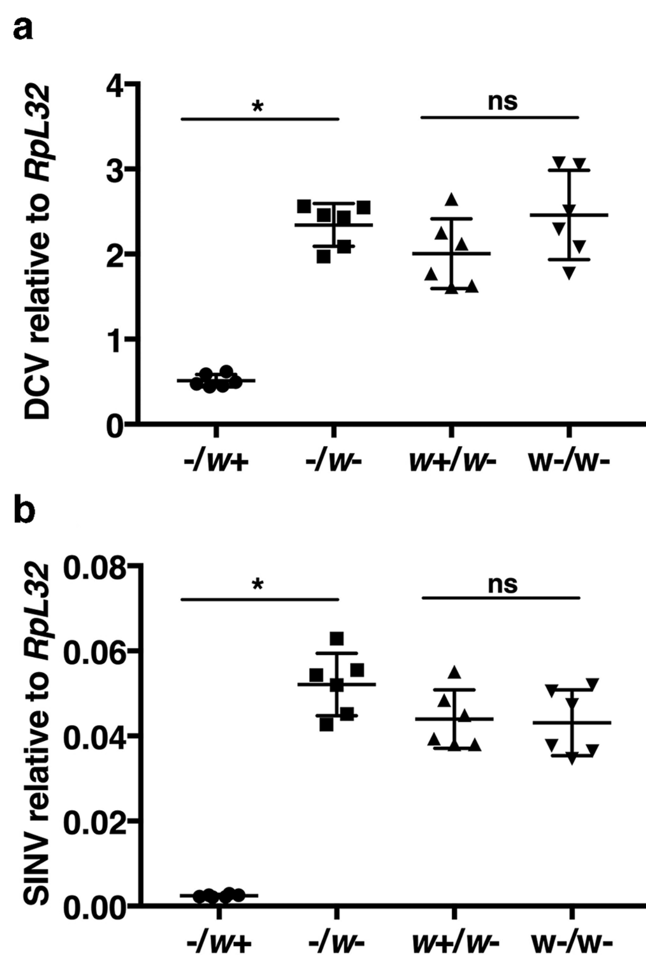

Fig. 3. Co-culture with *Wolbachia*-infected cells does not impact virus accumulation in *Wolbachia*-free cells. *Wolbachia*-infected (*w*+) and *Wolbachia*-free (*w*-) cells were physically separated from each other by a membrane with a pore size of 0.4 μm, as per Fig. 2a. This pore size allowed the transfer of virus between the cells in the top and the bottom of the transwell, but not the transfer of *Wolbachia*. Cells were co-cultured for 3 days before being infected with either (a) DCV or (b) SINV at an m.o.i. of 0.001 or an m.o.i. of 1, respectively. After 2 days of viral infection, cells in the bottom of the wells were harvested and viral accumulation was measured via RT-qPCR, using the *Drosophila* housekeeping gene *Rpl32* to normalize the data. Each dot represents a single-cell culture-assay well (for each treatment n=6, taken across three independent biological replicates), with mean represented by horizontal lines and standard deviation indicated. Welch's t-test was performed with Bonferonni correction for multiple comparisons and results were considered significant if they have a P-value of <0.05. Significance is indicated by asterisks (DCV-/*w*+, -/*w*- P<0.0002, *w*+/*w*-, *w*-/*w*- P=0.25; SINV -/*w*+, -/*w*- P<0.0002, *w*+/*w*-, *w*-/*w*- P=0.83).

cell. However, previous studies with Semliki Forest virus and SINV show that *Wolbachia* impacts virus replication, rather than entry into the cell [37, 40]. Taken together, this suggests that the viruses are capable of entering the cell in the presence of *Wolbachia*, and that *Wolbachia* protection involves interactions early in virus infection within the host cell. Therefore, the *Wolbachia* density of individual cells may be important in conferring protection.

Cell-autonomous protection has several implications for the possible mechanisms of antiviral protection. The results presented here suggest that an upregulation of systemic signalling throughout the organism is not likely to be the primary cause of the protection phenotype. Intercellular signalling, for example the release of antimicrobial peptides (AMP) and ROS into the cytoplasm of *Drosophila* cells, may play a role, and has indeed been correlated with antiviral protection in some species [45, 46], but this is likely to be a peripheral contribution to protection as it is not consistent across species. In a recent review [47], the inability of immune signalling and priming to explain the breadth of protection lead the authors to explore other mechanisms by which *Wolbachia* is interfering with the viral infection cycle. They analysed available transcriptomic data of mosquitoes during infection and found that the following gene families were altered: cellular trafficking, cytoskeleton, heat shock response, cell proliferation, chitin and cytochrome P450. Taken together with evidence that *Wolbachia* is mediating protection in the cell post viral entry [37], these gene families may be involved in protection and provide candidate pathways to be further explored.

It is also possible that *Wolbachia*-mediated antiviral protection involves a more passive mechanism. Cell-autonomous protection is consistent with the current hypothesis of competition between *Wolbachia* and virus for important host resources. There is some evidence to support this hypothesis in *Drosophila* [32], which found that competition for cholesterol contributes to antiviral protection, but could not be solely used to explain the protection. Most positive-sense RNA viruses hijack host-cell lipids in order to form a replication complex, which provides a favourable environment for replicative machinery and can even aid in hiding the virus from immune detection. Cholesterol is one lipid that is utilized in viral replication complexes, but there are a number of others that have been implicated in viral replication that have not been investigated in the context of *Wolbachia* infection and protection. Sequestering of lipids by *Wolbachia* may provide a partial explanation to what seems to be a multi-faceted mechanism of protection.

CONCLUSION

The results of this study are consistent with the protection seen in the whole organism *Drosophila melanogaster,* and with other *Wolbachia*-infected cell lines. We found that *Wolbachia* conferred protection against two viruses of different families, one of which is an arbovirus, and that this protection was dependent on *Wolbachia* infection within a cell culture. This finding suggests a focus on mechanisms involving intracellular signalling, or more passive means such as competition for resources between bacteria and virus.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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