Received: 19 July 2023 | Accepted: 22 February 2024

DOI: 10.1111/imb.12904

ORIGINAL ARTICLE

Insect Molecular Biology

Royal Entomological Society

Alpha-mannosidase-2 modulates arbovirus infection in a pathogen- and *Wolbachia*-specific manner in *Aedes aegypti* mosquitoes

Nadya Urakova[1] | Renuka E. Joseph[1,2,3] | Allyn Huntsinger[1] | Vanessa M. Macias[1] | Matthew J. Jones[1,2] | Leah T. Sigle[1,2] | Ming Li[4] | Omar S. Akbari[4] | Zhiyong Xi[5] | Konstantinos Lymperopoulos[6] | Richard T. Sayre[6] | Elisabeth A. McGraw[1,2,3] | Jason L. Rasgon[1,2,3]

[1]Department of Entomology, The Pennsylvania State University, University Park, Pennsylvania, USA

[2]The Center for Infectious Disease Dynamics, The Pennsylvania State University, University Park, Pennsylvania, USA

[3]The Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, Pennsylvania, USA

[4]Division of Biological Sciences, Section of Cell and Developmental Biology, University of California, San Diego, La Jolla, California, USA

[5]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan, USA

[6]Pebble Labs, Little Fly Division, Los Alamos, New Mexico, USA

Correspondence
Jason L. Rasgon, Department of Entomology, The Pennsylvania State University, University Park, PA, USA.
Email: jlr54@psu.edu

Present addresses
Nadya Urakova, Leiden University Medical Center, Leiden, The Netherlands; and Vanessa M. Macias, Department of Biological Sciences, University of North Texas, Denton, Texas, USA.

Funding information
Pebble Labs; National Institutes of Health, Grant/Award Numbers: R01AI116636, R01AI150251, R01AI143758, R01AI151004, DP2AI152071, R21AI156078; National Science Foundation, Grant/Award Number: 1645331; United States Agency for International Development, Grant/Award Number: AID-OAA-F-16-00082; Defense Advanced Research Projects Agency, Grant/Award Number: HR0011-17-2-0047; Dorothy Foehr Huck and J. Lloyd Huck Endowment

Associate Editor: Sassan Asgari

Abstract

Multiple *Wolbachia* strains can block pathogen infection, replication and/or transmission in *Aedes aegypti* mosquitoes under both laboratory and field conditions. However, *Wolbachia* effects on pathogens can be highly variable across systems and the factors governing this variability are not well understood. It is increasingly clear that the mosquito host is not a passive player in which *Wolbachia* governs pathogen transmission phenotypes; rather, the genetics of the host can significantly modulate *Wolbachia*-mediated pathogen blocking. Specifically, previous work linked variation in *Wolbachia* pathogen blocking to polymorphisms in the mosquito alpha-mannosidase-2 (αMan2) gene. Here we use CRISPR-Cas9 mutagenesis to functionally test this association. We developed αMan2 knockouts and examined effects on both *Wolbachia* and virus levels, using dengue virus (DENV; *Flaviviridae*) and Mayaro virus (MAYV; *Togaviridae*). *Wolbachia* titres were significantly elevated in αMan2 knockout (KO) mosquitoes, but there were complex interactions with virus infection and replication. In *Wolbachia*-uninfected mosquitoes, the αMan2 KO mutation was associated with decreased DENV titres, but in a *Wolbachia*-infected background, the αMan2 KO mutation significantly increased virus titres. In contrast, the αMan2 KO mutation significantly increased MAYV replication in *Wolbachia*-uninfected mosquitoes and did not affect *Wolbachia*-mediated virus blocking. These results demonstrate that αMan2 modulates arbovirus infection in *A. aegypti* mosquitoes in a pathogen- and *Wolbachia*-specific manner, and that *Wolbachia*-mediated

pathogen blocking is a complex phenotype dependent on the mosquito host genotype and the pathogen. These results have a significant impact for the design and use of *Wolbachia*-based strategies to control vector-borne pathogens.

KEYWORDS
alpha-mannosidase-2, *CRISPR*, dengue virus, Mayaro virus, *Wolbachia*

INTRODUCTION

Dengue virus (DENV) (genus *Flavivirus*, family *Flaviviridae*) is an important human pathogen that is transmitted primarily by *Aedes aegypti* mosquitoes (Bhatt et al., 2013). Mayaro virus (MAYV) (genus *Alphavirus*, family *Togaviridae*) is an emerging human pathogen that is transmitted mainly by *Haemagogus janthinomys* mosquitoes (Pujhari et al., 2022); however, *A. aegypti* mosquitoes are also competent vectors for this virus (Pereira et al., 2020). There are no approved vaccines or specific antivirals to prevent and manage disease outbreaks that are caused by either virus and thus novel strategies for disease control are needed to combat arbovirus infections. The use of the intracellular invertebrate-specific bacterium *Wolbachia* as a biological control agent against *A. aegypti* has emerged as an innovative vector control strategy to reduce arbovirus transmission. *Wolbachia* bacteria are useful because, when incorporated into *A. aegypti* mosquitoes, it suppresses vector populations via a reproductive manipulation called cytoplasmic incompatibility (CI; Beckmann et al., 2019; Caragata et al., 2021; Sicard et al., 2019) and also prevents replication of viruses inside mosquitoes, a trait known as pathogen blocking (PB), thereby limiting subsequent virus transmission to humans (Caragata et al., 2021).

Wolbachia-mediated PB phenotypes in mosquitoes depend not just on the infecting *Wolbachia* strain but also on many other factors including pathogen, infection type (natural vs. artificial), environmental conditions and, importantly, host genetics (Ford et al., 2019; Ford et al., 2020; Liang et al., 2022). For example, mosquitoes infected with the *w*AlbB *Wolbachia* strain exhibited better arbovirus blocking when the mosquito nuclear genome was derived from Singapore compared to Mexico-derived (Liang et al., 2022). Ford et al. found enough standing genetic variation in Australian *A. aegypti* to select for significant weakening of PB within a few generations of artificial selection, suggesting that the host genetic background can have a strong effect on PB (Ford et al., 2019). Identified candidate mosquito host genes for this modulation were not the canonical suspects of mosquito innate immunity or detoxification; rather, they were primarily related to cell adhesion, Notch signalling and cell cycle (Ford et al., 2019; Ford et al., 2020) highlighting our current lack of mechanistic understanding of the PB phenomenon.

Ford et al. identified single nucleotide polymorphisms in the non-coding region of the alpha-mannosidase-2 (αMan2) gene that were strongly associated with PB strength in *Wolbachia*-infected *A. aegypti* mosquitoes selected for high versus low *Wolbachia*-mediated PB of DENV (Ford et al., 2019). αMan2 is putatively involved in protein glycosylation (Nemčovičová et al., 2013), and thus could alter PB by modulating viral glycosylation. Protein glycosylation, the enzymatic attachment of oligosaccharide structures to the peptide backbone, is an important post-translational modification for both host cell and viral proteins (Rogers & Heise, 2009; Schiller et al., 2012; Yap et al., 2017). In eukaryotic cells, glycosylation is responsible for many functions, including proper protein folding, trafficking, stability, receptor–ligand recognition and cell adhesion (Schiller et al., 2012). Viruses do not have their own protein glycosylation machinery and employ host cellular enzymes for this purpose (Yap et al., 2017). Glycosylation of viral proteins plays a crucial role in the lifecycles of dengue and other viruses, influencing virus infectivity, pathogenicity and host immune responses (Rogers & Heise, 2009; Vigerust & Shepherd, 2007). Enzymes involved in protein glycosylation are important potential targets to control viral replication in eukaryotic cells (Chang et al., 2013; Pérez-García et al., 2017). However, how specific genes in these pathways affect arboviral replication in mosquito vectors is poorly understood.

We previously attempted to validate the role of αMan2 in wAlbB-mediated *Wolbachia* PB using RNAi but we were unable to knock down expression of this gene in *Wolbachia*-infected mosquitoes (Sigle et al., 2022). Therefore, in this study, we used CRISPR-Cas9 gene editing to ablate the αMan2 gene in *A. aegypti* and examined effects of gene knockout (KO) on mosquito vector competence for DENV and MAYV in both *Wolbachia*-infected and uninfected mosquitoes. Results demonstrated complicated interactions between gene KO, *Wolbachia* infection and viral pathogen, highlighting the complex nature of *Wolbachia* PB phenotypes.

MATERIALS AND METHODS

Cells

African green monkey kidney (Vero, ATCC CCL-81) cells were obtained from ATCC (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco/Thermo Fisher, Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS; Gibco/Thermo Fisher), 100 μg/mL of streptomycin (Gibco/Thermo Fisher) and 100 units/mL of penicillin (Gibco/Thermo Fisher) at 37°C in 5% CO_2. *Aedes albopictus* cells (C6/36) were obtained from Sigma-Aldrich, St. Louis, MO, USA, and maintained in RPMI 1640 medium (Gibco/Thermo Fisher) supplemented with 10% FBS (Gibco/Thermo Fisher), 100 μg/mL of streptomycin (Gibco/Thermo Fisher) and 100 units/mL of penicillin (Gibco/Thermo Fisher) at 28°C.

Viruses

MAYV strain BEAN343102 (GenBank: KP842802.1) was obtained from BEI Resources, NIAID, NIH (Manassas, VA, USA). To produce MAYV stocks, virus was propagated on Vero cells for 24 h and stored at −80°C. DENV serotype 2 strain JAM 1409 (Bennett et al., 2002) was propagated on C6/36 cells for 7 days as previously described (Terradas et al., 2017). MAYV stocks were initially quantified by plaque assay, while DENV stocks were initially quantified by qPCR. For all mosquito infection experiments, viruses were quantified by focus-forming assays (FFAs; see below for specific methods).

Antibodies

Mouse monoclonal anti-alphavirus antibodies (G77L; #MA5-18173) were obtained from Thermo Fisher and used in FFAs to detect MAYV at a dilution of 1:40 and incubated at 4°C overnight. Mouse monoclonal anti-flavivirus group antigen antibodies, clone D1-4G2-4-15 (produced in vitro; NR-50327), were obtained from BEI Resources, NIAID, NIH (Manassas, VA, USA). These antibodies were used in FFAs for the detection of DENV antigens at a dilution of 1:500 and incubated at 4°C overnight. Goat anti-mouse IgG (H + L) highly cross-adsorbed secondary antibodies, Alexa Fluor 488 (A-11029) were purchased from Invitrogen. Secondary antibodies were used in FFAs at a dilution of 1:1000 and incubated at a room temperature for at least 3 h or at 4°C overnight.

Plaque assay for the quantification of MAYV stocks

For quantification of MAYV viral stocks, Vero cells were seeded in 6-well plates at a density of 5×10^5 cells/well. Ten-fold serial dilutions of virus stocks were prepared in PBS and 200 µL of these dilutions were used for infections. Cells were infected for 1 h at 37°C, infectious media removed and cells covered with 1 mL of complete DMEM medium containing 0.5% agarose. Three days post-infection, cells were fixed with 4% formaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS; Gibco/Thermo Fisher) for 25 min, agarose covers were removed and cells were stained for 5 min using aqueous solution containing 1% crystal violet and 20% ethanol to visualise plaques. For mosquito experiments, virus was quantified by FFA (see below).

qPCR for the quantification of DENV stocks

Viral RNA was purified using Direct-zol RNA kit (Zymo Research) according to the manufacturer's instructions and used as template in qPCRs. All primer sequences are in Table S1. qPCRs were set up using TaqMan™ Fast Virus 1-Step Master Mix (Thermo Fisher) and run on an ABI 7500 Fast Real-time PCR System (Applied Biosystems/Thermo Fisher). The thermocycling conditions were as follows: 50°C for 5 min; 95°C for 20 s; 35 cycles of 95°C for 3 s; 60°C for 30 s; 72°C for 1 s and 40°C for 10 s. Product was detected by measuring the fluorescence signal from the FAM reporter. A standard reference curve of known quantities of a DENV-2 genomic fragment was used for absolute quantification by qPCR. The DENV-2 genomic fragment was inserted into a plasmid and transformed into *Escherichia coli* as previously described (Terradas et al., 2017). The linearised and purified fragment was serially diluted ranging from 10^7 to 10^2 copies and used to create a standard curve of DENV amplification. The standard curve was run in duplicate on each 96-well plate, and the limits of detection were set at 10^2 copies. For mosquito experiments, virus was quantified by FFA (see below).

Mosquito rearing

A. aegypti mosquitoes (Liverpool genetic background) expressing Cas9 protein in the germline (AAEL006511-Cas9; Li et al., 2017) were used. *A. aegypti* mosquitoes stably infected with the wAlbB strain of *Wolbachia* (backcrossed into the Merida, Mexico genetic background; Sigle et al., 2022) were provided by Prof. Zhiyong Xi, Michigan State University. Mosquitoes were reared at the PSU Millennium Sciences Complex insectary under the following environmental conditions: 27 ± 1°C, 12:12 h light:dark diurnal cycle, 80% relative humidity. For reproduction, mosquitoes were maintained on expired anonymous human blood using a 37°C water-jacketed membrane feeder. Larvae were fed on koi fish pellets (TetraPond). Adult mosquitoes were maintained on 10% sucrose solution.

Preparation of single guide RNAs

The αMan2 Entrez Gene ID 5564678 gene sequence was used as a reference to design single guide RNAs (sgRNAs) using CRISPOR (Concordet & Haeussler, 2018). sgRNAs were produced using overlapping nucleotides with the MegaScript T7 (Invitrogen/Thermo Fisher) in vitro transcription system. PCR templates for sgRNAs were produced using Phusion High-Fidelity DNA polymerase. The thermocycling conditions were as follows: 98°C for 20 s; 35 cycles of 98°C for 1 min s; 58°C for 1 min; 72°C for 1 min and a final extension of 72°C for 7 min. Oligonucleotide sequences are given in Table S1. PCR products were purified using NucleoSpin Gel and PCR Clean-Up kit (Takara Bio, Kusatsu, Shiga, Japan), and 600 ng-1 µg of DNA templates were added to set up in vitro transcription reactions. Reactions were run for 16 h at 37°C, treated with Turbo DNAse according to manufacturers' instructions and purified using the MegaClear column purification kit (Thermo Fisher). The purified sgRNAs were tested with an in vitro cleavage assay. To produce a DNA template, genomic DNA (gDNA) from *A. aegypti* mosquitoes was purified using E.Z.N.A. MicroElute Genomic DNA Kit (Omega Bio-tek, Norcross, GA, USA), and the target region was amplified using Phire Animal Tissue Direct PCR Kit (Thermo Fisher) as described below. Reactions containing DNA template, individual sgRNAs, and Cas9 protein in 1X

NEB 3.1 buffer (New England Biolabs, Ipswich, MA, USA) were incubated at 37°C for 2 h, and diagnostic bands were visualised by electrophoresis on 1% agarose gel. sgRNAs for *A. aegypti* embryo injections were used at concentrations ranging between 70 ng/μL and 180 μg/μL.

Embryo injections and establishment of KO mosquito lines

Four to five days after blood feeding, 5–10 mated *Wolbachia*-uninfected females were placed into a *Drosophila* vial with damp cotton and filter paper and placed in the dark for 50 min to stimulate oviposition. To generate heritable mutations in *A. aegypti* mosquitoes, mixtures of selected sgRNAs were injected into pre-blastoderm-stage embryos of Cas9-expressing mosquitoes 1–2 h after laying. Briefly, embryos were aligned (with posterior poles on one side) on damp filter paper using a paintbrush, transferred on a glass slide using double-sided scotch tape, dried for 1 min and covered with a mixture of Halocarbon 700 oil and Halocarbon 27 oil (1:1) to prevent further desiccation. Embryos were injected into the posterior poles with quartz needles (QF100-70-10, Sutter Instrument, Novato, CA, USA) pulled by a Sutter P2000 needle puller (program 50, HEAT = 500, FIL = 5, VEL = 50, DEL = 128, PUL = 0), using a Femtojet injector (Eppendorf, Hamburg, Germany) and an InjectMan micromanipulator using the following settings: injection pressure (pi) 1000 hPa, compensation pressure (pc) 700 hPa, injection time (manual mode) 2–3 s. After injection, embryos were transferred on the damp filter paper into egg cups with wet cotton, kept in the humid insectary for 4–5 days and then hatched. Injected embryos (G_0) that hatched and survived until adulthood were crossed individually. Legs of G_1 mosquitoes were individually screened by PCR for the presence of deletions in the target gene as described below. A single heterozygous founder mosquito was outcrossed with wild-type age-matched *A. aegypti* mosquitoes to establish a KO mosquito line (see Results). As the target gene was located in the chromosome 1, the mutation was sex-linked (Hall et al., 2015). As a result, to obtain homozygous mutants of both sexes, the selection process relied on chromosome recombination and identification of recombinant mosquitoes.

Mosquito screenings for mutations

To screen live mosquitoes for the presence of deletions in the target gene, Phire Animal Tissue Direct PCR Kit (Thermo Fisher) was used according to manufacturers' instructions. Briefly, mosquitoes were anaesthetised on ice, a leg from each mosquito was removed using sharp forceps and immersed into 20 μL of sample dilution buffer supplemented with 0.5 μL of DNA release reagent. Leg samples in dilution buffer were incubated for 3 min at 98°C and then used in PCR reactions. Primer sequences are provided in Table S1.

To characterise the αMan2 mutation at the transcript level, total RNA from αMan2 KO and wild-type mosquitoes was purified using E.Z.N.A. Total RNA kit (Omega Bio-tek) and cDNA synthesised using a gene-specific reverse primer and SuperScript III First-Strand Synthesis System (Thermo Fisher) according to manufacturers' instructions. For cDNA synthesis, negative control gDNA from KO and wild-type mosquitoes was purified using E.Z.N.A MicroElute Genomic DNA Kit (Omega Bio-tek). PCR reactions were performed using Phire Animal Tissue Direct PCR Kit as described above. Information on primer sequences is in Table S1. For the detection of deletions in target genes, PCR products were separated by 2% agarose gel electrophoresis. Samples that separated into multiple bands were considered likely to contain an indel. The presence of αMan2 deletion(s) in both DNA and mRNA was then confirmed by PCR and direct sequencing of the target region.

Generation of Wolbachia-infected lines

Wolbachia-infected *A. aegypti* females were crossed with KO *Wolbachia*-negative male mosquitoes. Homozygous mutant *Wolbachia*-infected males and heterozygous *Wolbachia*-infected females were isolated and further crossed to obtain homozygous *Wolbachia*-infected male and female mosquitoes. Homozygous mutant *Wolbachia*-infected male and female mosquitoes were isolated crossed to establish a pure homozygous *Wolbachia*-infected αMan2 KO mosquito line. *Wolbachia*-negative male mosquitoes from the parental Cas9-expressing *A. aegypti* line, which was used for embryo injections, were crossed with *Wolbachia*-infected *A. aegypti* females to obtain a *Wolbachia*-infected wild-type control line with comparable genetic background for infection experiments.

Quantification of relative Wolbachia density

Total DNA was extracted from *Wolbachia*-infected mosquito homogenates (females, 7 days post-bloodfeed; 11–12 days post-emergence) using a E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek) kit according to the manufacturer's instructions. qPCR was performed using PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA, USA) on a Rotor-Gene Q qPCR machine (Qiagen, Hilden, Germany) under the following thermocycling conditions: 95°C for 2 min for initial denaturation; 40 cycles at 95°C for 10 s, 60°C for 40 s, 72°C for 30 s for DNA amplification and data acquisition; 55–99°C (5 s per increment) for the melt curve analysis. Relative *Wolbachia* densities were obtained by normalising *Wolbachia* titres to the RpS17 gene levels as described previously (Ford et al., 2019). Primer sequences are provided in Table S1. Crosses to introgress KO mutations into the *Wolbachia*-infected background are described in Results and Figure 1c.

Vector competence studies

Four- to five-day-old female mosquitoes were blood fed for approximately 1 h on infected human blood containing 10^7 infectious MAYV

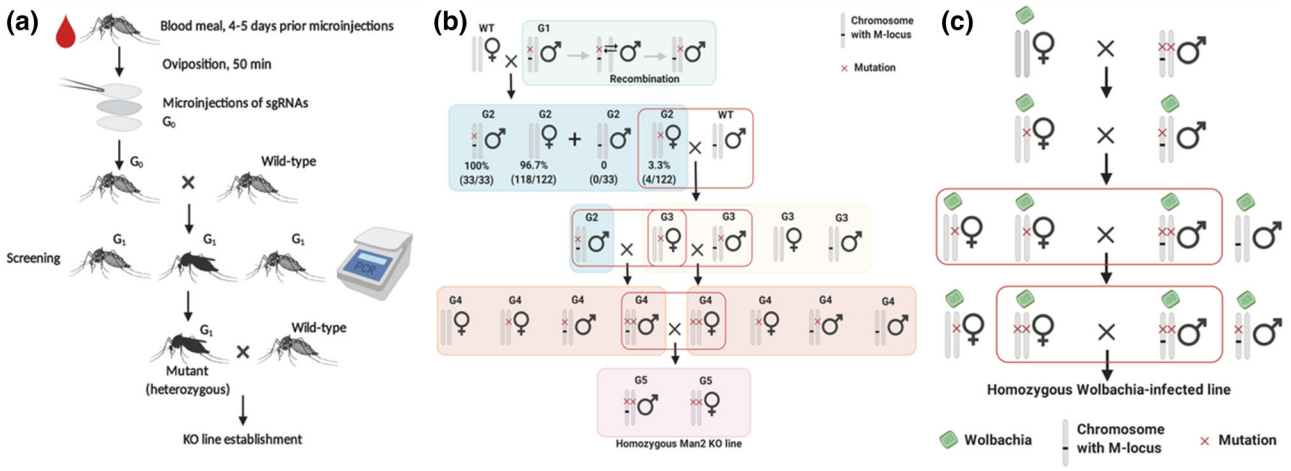

FIGURE 1 Overview of the approach used to generate *A. aegypti* strains used in this study. (a) Generation of αMan2 KO mutations in *Wolbachia*-uninfected mosquitoes using CRISPR-Cas9 mutagenesis. (b) Crossing scheme to generate a homozygous αMan2 KO line. (c) Crossing scheme to generate a homozygous αMan2 KO line infected with *Wolbachia*.

particles per mL or 10^6 infectious DENV particles per mL. After blood feeding, mosquitoes were anaesthetised on ice and fully engorged females were transferred into cardboard cages; unfed females were discarded. Seven days post-infection, mosquitoes were anaesthetised using triethylamine (Sigma-Aldrich) and processed for vector competence assays. Although the KO line was fertile and viable, the mosquitoes were very fragile and died easily, and thus we were not able to examine timepoints beyond 7 days. Mosquitoes were forced to salivate for 30 min into glass capillaries filled with a mix of 50% sucrose solution and FBS (1:1) to collect saliva samples. Body (infection), legs (dissemination) and saliva (transmission) were then separately immersed in diluent solution containing 10% of FBS, 100 μg/mL of streptomycin, 100 units/mL of penicillin, 50 μg/mL gentamicin and 2.5 μg/mL Amphotericin B in PBS. Body and legs samples were further homogenised by a single zinc-plated, steel, 4.5 mm bead using TissueLyser II (Qiagen) at 30 Hz for 2 min and centrifuged at 2000 g at 4°C for 7 min in a bench top centrifuge to clear the homogenates. Samples were stored at −80°C. Virus titres in collected samples were determined by FFAs.

FFA for the quantification of MAYV and DENV

FFA was used to quantify both DENV and MAYV in all mosquito infection experiments. Vero or C6/36 cells were seeded in 96-well plates at a density of 3×10^4 cells/well or 3×10^5 cells/well for the titration of MAYV or DENV, respectively. Ten-fold serial dilutions (in serum-free medium) of virus samples obtained from mosquito bodies and legs were prepared and 30 μL of each were used in assays. Saliva samples were not further diluted. Cells were infected for 1 h at 37 or 28°C for MAYV and DENV assays, respectively. Infectious solutions were then removed and cells covered with 100 μL of complete growth medium (DMEM or RPMI) containing 0.8% methylcellulose (Sigma-Aldrich) and incubated at their respective temperatures. After 24 h for MAYV or 3 days for DENV assays, overlay medium was removed, cells were fixed with 4% formalin (Sigma-Aldrich) in PBS (Gibco/Thermo Fisher) for 15 min and permeabilised with 0.2% TritonX in PBS for 15 min. Primary antibodies were diluted in PBS and incubated overnight at 4°C. Secondary antibodies were incubated overnight at 4°C for MAYV assays and 3 h at room temperature for DENV. After the final wash, cells were dried briefly, and MAYV or DENV foci immediately counted using an Olympus BX41 inverted microscope equipped with an UPlanFI 4X objective and a FITC filter. Dissemination rates only included mosquitoes that became infected. Transmission rates only included mosquitoes that became disseminated.

Statistical analysis

All experiments were replicated twice. Infection, dissemination and transmission rates were analysed using contingency tables. Data on *Wolbachia* titres were analysed by Mann–Whitney U tests. Due to violation of the equal variance assumption, data on viral titres were analysed using the Brown, Forsythe ANOVA method with Welch's correction for multiple tests.

RESULTS

Generation of Wolbachia-negative and Wolbachia-positive αMan2 KO *A. aegypti* mosquitoes

To generate a deletion in the αMan2 gene, *Wolbachia*-uninfected *A. aegypti* embryos expressing Cas9 protein (G_0, $N = 115$) were injected with a mix of four sgRNAs targeting exon 5 of the gene. Surviving G_0 individuals (females $N = 19$, males $N = 10$) were outcrossed to wild-type mates (1 male per 1–2 females), females blood fed, eggs

collected and hatched in small batches for further screening (Figure 1a). Forty G_1 male mosquitoes were individually screened by PCR for the presence of deletions in the target gene. Three G_1 males with αMan2 deletions were identified: two with an identical 13 nt deletion and one with a double deletion allele consisting of a 46 nt deletion at one sgRNA target site and a 9 nt deletion at another sgRNA target site (55 nt deletion total) (Figure S1A). This 55 nt deletion was predicted to result in a 155-amino acid-long truncated protein instead of a 1174-amino acid long functional enzyme. The male mosquito with two deletions (totalling 55 nt) was further crossed with 7 age-matched wild-type females to establish a line (Figure 1a).

Since αMan2 is located in on chromosome 1, deletions in this gene were expected to be sex linked [19]. All G_2 male progeny from the selected heterozygous G_1 mutant mosquito that were screened ($N = 33$) carried deletions, while the majority of G_2 females were wild-type. To obtain mutant females, we relied on identification of recombinant mosquitoes. Four out of 122 screened G_2 females (3.3%) were recombinants and carried a deleted copy of αMan2. These G_2 females were further crossed with wild-type males to obtain G_3 males with the mutation on the opposite chromosome. Homozygous mutant males were obtained via crossing G_3 mutant females and the generated G_2 mutant males. Homozygous mutant males and females were screened, selected and crossed to obtain a homozygous αMan2 KO line (Figure 1b). The presence of αMan2 deletions in both DNA and mRNA was confirmed by PCR and direct sequencing of the target region (Figure S1B).

To generate a *Wolbachia*-infected αMan2 KO line, *Wolbachia*-infected *A. aegypti* females we crossed with αMan2 KO *Wolbachia*-negative male mosquitoes so that CI would not sterilise the cross (Beckmann et al., 2019). Every generation after crossing was checked by PCR for the presence of both the mutation and *Wolbachia* infection, and heterozygous *Wolbachia*-infected males and females were crossed. Homozygous *Wolbachia*-infected males and heterozygous *Wolbachia*-infected females were selected and further crossed as described above to obtain homozygous *Wolbachia*-infected male and female mosquitoes. Homozygous *Wolbachia*-infected male and female mosquitoes were selected using PCR and crossed to establish a pure homozygous *Wolbachia*-infected αMan2 KO mosquito line (Figure 1c). *Wolbachia*-negative male mosquitoes from the parental Cas9-expressing *A. aegypti* line, which was used for embryo injections, were crossed with *Wolbachia*-infected *A. aegypti* females following similar procedure as described above to obtain a *Wolbachia*-infected wild-type control line with comparable genetic background for infection experiments.

Effect of αMan2 KO on Wolbachia titres in *A. aegypti* mosquitoes

Mosquitoes carrying the αMan2 KO mutation had mean *Wolbachia* levels that were approximately 2-fold increased compared to the wild-type genetic background (Mann–Whitney *U* test, $p = 0.0007$; Figure 2).

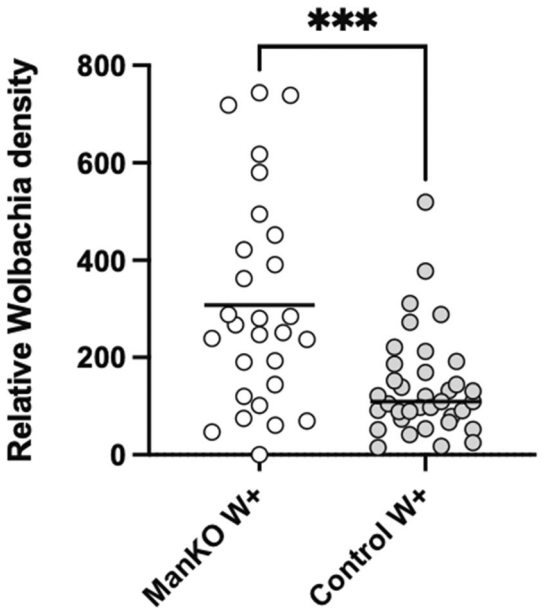

FIGURE 2 *Wolbachia* titres in αMan2 KO ($N = 28$) and wild-type ($N = 36$) mosquitoes. Mutant mosquitoes had significantly higher levels of *Wolbachia* compared to wild type (Mann–Whitney *U* test, $p = 0.0007$).

Effect of Wolbachia and αMan2 KO on DENV infection, dissemination and transmission rates

DENV infection and dissemination rates were lower in *Wolbachia*-infected wild-type mosquitoes compared to Wolbachia-uninfected wild-type mosquitoes (50% vs. 36% infection) although this trend was not statistically significant (Table 1). In *Wolbachia*-uninfected mosquitoes, the αMan2 KO mutation was associated with significantly reduced DENV infection rates (16% vs. 50%; Table 1). Although either *Wolbachia* alone or the αMan2 KO mutation alone both tended to reduce DENV infection and dissemination rates, the two effects were not additive. Rather, there was an interaction between *Wolbachia* infection status and genotype; when the αMan2 KO mutation was present in a *Wolbachia*-infected background, infection rates were similar to *Wolbachia*-uninfected wild-type mosquitoes (50% vs. 46%; $p < 0.05$; Table 1). We did not observe DENV transmission in any treatment, possibly due to the timepoint assessed.

Effect of Wolbachia and αMan2 KO on DENV titres in mosquitoes

In a wild-type genetic background, DENV titres were significantly lower in *Wolbachia*-infected mosquitoes compared to uninfected (Figure 3a); a demonstration of canonical *Wolbachia*-induced PB. In a *Wolbachia*-uninfected background, mosquitoes with the αMan2 KO mutation had reduced DENV titres (Figure 3a). We observed an interaction between *Wolbachia* infection status and genotype, where the αMan2 KO mutation in a *Wolbachia*-infected background reduced the

TABLE 1 Virus infection, dissemination and transmission rates for experimental treatments 7 days post-infection.

Virus	Genotype	*Wolbachia*	*N*	Infection	Sig	Dissemination	Sig	Transmission	Sig
DENV	WT	No	36	0.50	a	0.22	a	0.00	a
DENV	WT	Yes	69	0.36	a	0.07	a,b	0.00	a
DENV	Man2 KO	No	37	0.16	b	0.03	a,b	0.00	a
DENV	Man2 KO	Yes	46	0.46	a	0.02	b,c	0.00	a
MAYV	WT	No	71	1.00	a	1.00	a	0.42	a
MAYV	WT	Yes	64	0.45	b	0.19	b	0.00	c
MAYV	Man2 KO	No	43	0.98	a	0.95	a	0.33	b,c
MAYV	Man2 KO	Yes	66	0.27	c	0.08	b	0.00	c

Note: Treatments with different letters are significantly different (contingency table analysis; $p < 0.05$).

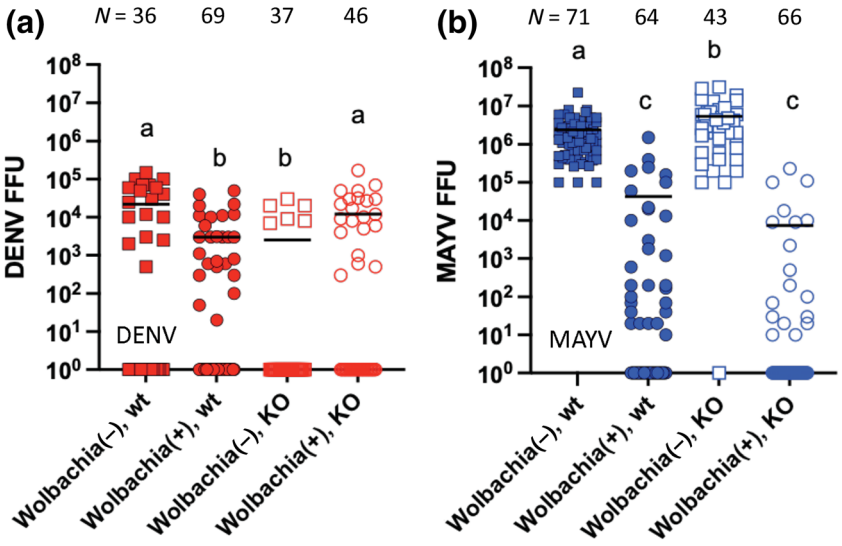

FIGURE 3 (a) DENV and (b) MAYV body titres in experimental mosquitoes 7 days post-infection. Red = DENV; Blue = MAYV; Squares = *Wolbachia*-uninfected; Circles = *Wolbachia*-infected; Filled symbols = wild-type; open symbols = αMan2 KO. Viruses were analysed separately; treatments with different letters are significantly different (Brown–Forsythe ANOVA with Welch's correction; $p < 0.01$). Data are pooled from two replicate experiments. *N* = sample size.

ability for *Wolbachia* to suppress DENV (Figure 3a). DENV dissemination titres in mosquito legs between treatments did not significantly differ, possibly due to lower dissemination rates and resulting lack of power to detect a statistical difference (Figure S2A).

Effect of Wolbachia and αMan2 KO on MAYV infection, dissemination and transmission rates

MAYV infection and dissemination rates were higher generally compared to DENV, perhaps due to higher initial viral titres or greater viral permissiveness. 100% of wild-type, *Wolbachia*-uninfected mosquitoes became infected with and disseminated MAYV. *Wolbachia*-infected, wild-type mosquitoes had significantly reduced infection (45%) and dissemination (19%) rates as would be expected from *Wolbachia*-induced PB (Table 1, $p < 0.05$). In the absence of *Wolbachia*, infection and dissemination rates were similar in αMan2 KO and wild-type mosquitoes. αMan2 KO, *Wolbachia*-infected mosquitoes had the lowest infection (27%) and dissemination (8%) rates, opposite to what was observed for DENV (Table 1). We did observe transmission of MAYV in these experiments, where the highest transmission rate (42%) was observed in wild-type *Wolbachia*-uninfected mosquitoes, and no transmission was observed in *Wolbachia*-infected mosquitoes, regardless of genotype (Table 1). *Wolbachia* uninfected KO mosquitoes had intermediate transmission rates (33%; Table 1).

Effect of Wolbachia and αMan2 KO on MAYV titres in mosquitoes

In a wild-type genetic background, MAYV titres were significantly lower in *Wolbachia*-infected mosquitoes compared to uninfected (Figure 3b); again consistent with canonical *Wolbachia*-induced PB. However, in a *Wolbachia*-uninfected background, the αMan2 KO

mutation was associated with enhanced MAYV titres compared to wild-type mosquitoes (Figure 3b). In a *Wolbachia*-infected background, the αMan2 KO mutation did not affect PB, and MAYV titres were indistinguishable from *Wolbachia*-infected wild-type mosquitoes (Figure 3b). MAYV dissemination titres in mosquito legs between treatments significantly differed in a similar pattern to body titres (Figure S2B).

DISCUSSION

The role of the mosquito nuclear genome in modulating *Wolbachia*-induced PB has been observed both empirically (Liang et al., 2022) and experimentally (Ford et al., 2019). A recent genetic screen identified that single-nucleotide polymorphisms in the *A. aegypti* αMan2 gene were associated with stronger or weaker *Wolbachia* (wMel)-mediated PB of DENV (Ford et al., 2019), but the functional role of this gene in DENV blocking remains unclear. Due to the intronic location of the identified polymorphisms, it was hypothesised that they could affect gene expression or splicing; however, no significant differences in αMan2 expression were found between selected low and high blocking mosquito populations (Ford et al., 2019). We recently published a study (Sigle et al., 2022) using RNAi to knock down expression of αMan2 in *Wolbachia* infected and uninfected *A. aegypti* to examine its effect on PB for DENV and Chikungunya virus (CHIKV); an alphavirus closely related to MAYV (Brustolin et al., 2018; Terradas et al., 2022). RNAi demonstrated some influence of αMan2 in virus infection in *Wolbachia*-negative mosquitoes, but we were unable to successfully knockdown gene expression in *Wolbachia*-infected mosquitoes and thus could not directly test the interaction of αMan2 and *Wolbachia* on virus infection (Sigle et al., 2022). To address this issue, here we used CRISPR-Cas9 gene editing to generate αMan2 KO mutations in *A. aegypti* mosquitoes to functionally investigate the role of this gene in arbovirus replication and found that the αMan2 KO mutation affected arboviruses in a pathogen and *Wolbachia* infection-specific manner. This is especially interesting as the *Wolbachia* strain used in the original genetic screen (Ford et al., 2019) was *w*Mel (originally from *Drosophila melanogaster*), while we performed experiments (here, and previously [Sigle et al., 2022]) using the *Wolbachia* strain *w*AlbB (originally from *Ae. albopictus*). These two *Wolbachia* strains are not closely related, yet both seem to interact with αMan2, suggesting that candidate genes identified by Ford et al. (2019) may be broadly applicable across different *Wolbachia* strains.

Differences in viral phenotypes between mutant and wild-type mosquitoes in a *Wolbachia*-infected background cannot be explained by a direct effect of the KO mutation on *Wolbachia* titres. *Wolbachia* levels were approximately twice as high in αMan2 KO mosquitoes compared to wild type. While *Wolbachia*-induced suppression of MAYV was similar in both αMan2 KO and wild-type mosquitoes, DENV was not blocked in *Wolbachia*-infected mutant mosquitoes, highlighting the complex interactions between the mosquito genome, *Wolbachia* and the specific viral pathogen. *Wolbachia* loads can be affected by mosquito immunity (Pan et al., 2018), and the mosquito immune system can be modulated by glycosylation pathways (Bednarska et al., 2017), suggesting a potential explanation for higher *Wolbachia* titres in αMan2 KO mosquitoes, although this phenomenon requires further study.

For DENV, the αMan2 KO mutation itself conferred some resistance to virus, significantly reducing viral titre. *Wolbachia* alone also reduced viral titre. However, there was an interaction between αMan2 genotype and *Wolbachia* infection; when the mutation was coupled with *Wolbachia* in the mosquito, DENV infections were no longer suppressed. We were not able to make conclusions on the effect of the mutation or *Wolbachia* on DENV transmission as no mosquitoes transmitted DENV at the studied timepoint of 7 days post-infection (due to the fragile nature of the KO line, mosquitoes generally did not survive long enough to examine later timepoints). We observed a different phenomenon with MAYV. In a *Wolbachia*-uninfected background, the αMan2 KO mutation did not significantly alter viral infection rates but did significantly enhance viral titres in the mosquito. In a *Wolbachia*-infected background, the mutation increased the ability for *Wolbachia* to suppress viral infection rates and did not interfere with the ability for *Wolbachia* to suppress viral titres, although it did not further enhance *Wolbachia* PB. As MAYV is transmitted faster than DENV, we were able to measure an effect of both *Wolbachia* and the mutation on MAYV transmission at 7 days post-infection.

The fact that the αMan2 mutation (in the absence of *Wolbachia*) had different effects on DENV versus MAYV is not necessarily surprising, as DENV is a flavivirus, while MAYV is an alphavirus. These two viral families are not closely related, and it has been demonstrated that the mosquito immune system responds differently to diverse viral groups (Samuel et al., 2018). The fact that the αMan2 KO mutation can have different effects on how *Wolbachia* suppresses different viral families is perhaps also not surprising, as *Wolbachia* has been shown to differentially suppress different pathogens in other systems (Fraser et al., 2020; Hughes et al., 2011; Hughes et al., 2012). Ultimately, these data demonstrate the complexity of the *Wolbachia* PB phenotype. In their screens, Ford et al. (2019, 2020) identified dozens of potential candidate genes regulating PB; here we have disrupted one of them. It is likely that disruption of other candidates could have equally complex consequences, to say nothing of multiple stacked mutations.

While our data show that *A. aegypti* αMan2 is a modulator of arbovirus infection, and likely involved in the *Wolbachia* PB phenotype, the mechanism by which it works, and has variable effects on different viruses, remains unclear. αMan2 is involved in protein glycosylation (Nemčovičová et al., 2013), which may affect viral biogenesis, replication and infectivity (Rogers & Heise, 2009), so it is logical that disruption of this gene would affect viral infection phenotypes. However, CRISPR is a blunt tool, and further molecular research is necessary to determine the specific mechanism by which αMan2 modulates replication of specific viruses and how it contributes to *Wolbachia* PB. It also should be noted that although all mosquito treatment strains used in this study were siblings, they differed in mitochondrial

background between *Wolbachia*-infected and uninfected lines and were not 100% homogeneous in their nuclear backgrounds.

AUTHOR CONTRIBUTIONS

Nadya Urakova: Conceptualization; investigation; writing – original draft; methodology; writing – review and editing; formal analysis; data curation. **Renuka E. Joseph:** Investigation. **Allyn Huntsinger:** Investigation. **Vanessa M. Macias:** Investigation. **Matthew J. Jones:** Investigation. **Leah T. Sigle:** Investigation. **Ming Li:** Investigation. **Omar S. Akbari:** Investigation; funding acquisition; supervision; writing – review and editing. **Zhiyong Xi:** Investigation; funding acquisition; writing – review and editing; resources. **Konstantinos Lymperopoulos:** Conceptualization; writing – review and editing. **Richard T. Sayre:** Conceptualization; writing – review and editing. **Elisabeth McGraw:** Conceptualization; funding acquisition; writing – review and editing; supervision. **Jason L. Rasgon:** Conceptualization; funding acquisition; writing – original draft; writing – review and editing; formal analysis; project administration; supervision.

ACKNOWLEDGEMENTS

We would like to thank Dr. Duverney Chaverra-Rodriguez for valuable advice concerning CRISPR experiments and Hillery Metz for valuable comments on a draft version of the manuscript.

FUNDING INFORMATION

This research was supported by NIH grants R01AI116636 and R01AI150251, NSF grant 1645331, and funds from the Dorothy Foehr Huck and J. Lloyd Huck endowment to JLR, NIH grant R01AI143758 to EAM, USAID grant AID-OAA-F-16-00082 to ZX, NIH grants R01AI151004, DP2AI152071, and R21AI156078, and DARPA Safe Genes Program Grant HR0011-17-2-0047 to OSA, and by a grant from Pebble Labs, Inc. to JLR, EAM, and ZX.

CONFLICT OF INTEREST STATEMENT

The authors declare that no conflict of interest exists.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the manuscript and in the supplementary material of this article.

ORCID

Jason L. Rasgon https://orcid.org/0000-0002-4050-8429

REFERENCES

Beckmann, J.F., Bonneau, M., Chen, H., Hochstrasser, M., Poinsot, D., Mercot, H. et al. (2019) The toxin-antidote model of cytoplasmic incompatibility: genetics and evolutionary implications. *Trends in Genetics*, 35, 175–185.

Bednarska, N.G., Wren, B.W. & Willcocks, S.J. (2017) The importance of the glycosylation of antimicrobial peptides: natural and synthetic approaches. *Drug Discovery Today*, 22, 919–926.

Bennett, K.E., Olson, K.E., de Munoz, L.M., Fernandez-Salas, I., Farfan-Ale, J.A., Higgs, S. et al. (2002) Variation in vector competence for dengue 2 virus among 24 collections of *Aedes aegypti* from Mexico and the United States. *The American Journal of Tropical Medicine and Hygiene*, 67, 85–92.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L. et al. (2013) The global distribution and burden of dengue. *Nature*, 496, 504–507.

Brustolin, M., Pujhari, S., Henderson, C.A. & Rasgon, J.L. (2018) Anopheles mosquitoes may drive invasion and transmission of Mayaro virus across geographically diverse regions. *PLoS NTD*, 12(11), e0006895.

Caragata, E.P., Dutra, H.L.C., Sucupira, P.H.F., Ferreira, A.G.A. & Moreira, L.A. (2021) Wolbachia as translational science: controlling mosquito-borne pathogens. *Trends in Parasitology*, 37, 1050–1067.

Chang, J., Block, T.M. & Guo, J.T. (2013) Antiviral therapies targeting host ER alpha-glucosidases: current status and future directions. *Antiviral Research*, 99, 251–260.

Concordet, J.P. & Haeussler, M. (2018) CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Research*, 46, W242–W245.

Ford, S.A., Albert, I., Allen, S.L., Chenoweth, S.F., Jones, M., Koh, C. et al. (2020) Artificial selection finds new hypotheses for the mechanism of Wolbachia-mediated dengue blocking in mosquitoes. *Frontiers in Microbiology*, 11, 1456.

Ford, S.A., Allen, S.L., Ohm, J.R., Sigle, L.T., Sebastian, A., Albert, I. et al. (2019) Selection on *Aedes aegypti* alters *Wolbachia*-mediated dengue virus blocking and fitness. *Nature Microbiology*, 4, 1832–1839.

Fraser, J.E., O'Donnell, T.B., Duyvestyn, J.M., O'Neill, S.L., Simmons, C.P. & Flores, H.A. (2020) Novel phenotype of *Wolbachia* strain wPip in *Aedes aegypti* challenges assumptions on mechanisms of *Wolbachia*-mediated dengue virus inhibition. *PLoS Pathogens*, 16, e1008410.

Hall, A.B., Basu, S., Jiang, X., Qi, Y., Timoshevskiy, V.A., Biedler, J.K. et al. (2015) A male-determining factor in the mosquito *Aedes aegypti*. *Science*, 348, 1268–1270.

Hughes, G.L., Koga, R., Xue, P., Fukatsu, T. & Rasgon, J.L. (2011) Wolbachia infections are virulent and inhibit the human malaria parasite plasmodium falciparum in *Anopheles gambiae*. *PLoS Pathogens*, 7(5), e1002043.

Hughes, G.L., Vega-Rodriguez, J., Xue, P. & Rasgon, J.L. (2012) Wolbachia strain wAlbB enhances infection by the rodent malaria parasite plasmodium berghei in *Anopheles gambiae* mosquitoes. *Applied and Environmental Microbiology*, 78, 1491–1495.

Li, M., Bui, M., Yang, T., Bowman, C.S., White, B.J. & Akbari, O.S. (2017) Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America*, 114, E10540–E10549.

Liang, X., Tan, C.H., Sun, Q., Zhang, M., Wong, P.S.J., Li, M.I. et al. (2022) Wolbachia wAlbB remains stable in *Aedes aegypti* over 15 years but exhibits genetic background-dependent variation in virus blocking. *Proceedings of the National Academy of Sciences of the United States of America*, 1(4), pgac203.

Nemčovičová, I., Šesták, S., Rendić, D., Plšková, M., Mucha, J., Wilson, I. et al. (2013) Characterisation of class I and II α-mannosidases from *Drosophila melanogaster*. *Glycoconjugate Journal*, 30, 899–909.

Pan, X., Pike, A., Joshi, D., Bian, G., McFadden, M.J., Lu, P. et al. (2018) The bacterium Wolbachia exploits host innate immunity to establish a symbiotic relationship with the dengue vector mosquito *Aedes aegypti*. *The ISME Journal*, 12, 277–288.

Pereira, T.N., Carvalho, F.D., De Mendonça, S.F., Rocha, M.N. & Moreira, L.A. (2020) Vector competence of *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* mosquitoes for Mayaro virus. *PLoS Neglected Tropical Diseases*, 14, e0007518.

Pérez-García, L.A., Martínez-Duncker, I. & Mora Montes, H.M. (2017) The endoplasmic reticulum alpha-glycosidases as potential targets for virus control. *Current Protein & Peptide Science*, 18, 1090–1097.

Pujhari, S., Brustolin, M., Heu, C.C., Smithwick, R., Larrosa, M., Hafenstein, S. et al. (2022) Characterization of Mayaro virus (strain

BeAn343102) biology in vertebrate and invertebrate cellular backgrounds. *The Journal of General Virology*, 103(10), 1794. Available from: https://doi.org/10.1099/jgv.0.001794

Rogers, K.M. & Heise, M. (2009) Modulation of cellular tropism and innate antiviral response by viral glycans. *Journal of Innate Immunity*, 1, 405–412.

Samuel, G.H., Adelman, Z.N. & Myles, K.M. (2018) Antiviral immunity and virus-mediated antagonism in disease vector mosquitoes. *Trends in Microbiology*, 26, 447–461.

Schiller, B., Hykollari, A., Yan, S., Paschinger, K. & Wilson, I.B.H. (2012) Complicated N-linked glycans in simple organisms. *Biological Chemistry*, 393, 661–673.

Sicard, M., Bonneau, M. & Weill, M. (2019) Wolbachia prevalence, diversity, and ability to induce cytoplasmic incompatibility in mosquitoes. *Current Opinion in Insect Science*, 34, 12–20.

Sigle, L.T., Jones, M., Novelo, M., Ford, S.A., Urakova, N., Lymperopoulos, K. et al. (2022) Assessing *Aedes aegypti* candidate genes during viral infection and Wolbachia-mediated pathogen blocking. *Insect Molecular Biology*, 31(3), 356–368.

Terradas, G., Joubert, D.A. & McGraw, E.A. (2017) The RNAi pathway plays a small part in Wolbachia-mediated blocking of dengue virus in mosquito cells. *Scientific Reports*, 7, 43847.

Terradas, G., Novelo, M., Metz, H., Brustolin, M. & Rasgon, J.L. (2022) Anopheles albimanus is a potential alphavirus vector in the Americas. *The American Journal of Tropical Medicine and Hygiene*, 108, 412–423. Available from: https://doi.org/10.4269/ajtmh.22-0417

Vigerust, D.J. & Shepherd, V.L. (2007) Virus glycosylation: role in virulence and immune interactions. *Trends in Microbiology*, 15, 211–218.

Yap, S.S.L., Nguyen-Khuong, T., Rudd, P.M. & Alonso, S. (2017) Dengue virus glycosylation: what do we know? *Frontiers in Microbiology*, 8, 1415.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1. Supporting Information.

How to cite this article: Urakova, N., Joseph, R.E., Huntsinger, A., Macias, V.M., Jones, M.J., Sigle, L.T. et al. (2024) Alpha-mannosidase-2 modulates arbovirus infection in a pathogen- and *Wolbachia*-specific manner in *Aedes aegypti* mosquitoes. *Insect Molecular Biology*, 1–10. Available from: https://doi.org/10.1111/imb.12904