A wMel *Wolbachia* variant in *Aedes aegypti* from field-collected *Drosophila melanogaster* with increased phenotypic stability under heat stress

Short title: wMel variants in *Aedes aegypti*

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Summary

Mosquito-borne diseases such as dengue, Zika and chikungunya remain a major cause of morbidity and mortality across tropical regions. Population replacement strategies involving the wMel strain of *Wolbachia* are being used widely to control mosquito-borne diseases transmitted by *Aedes aegypti*. However, these strategies may be influenced by environmental temperature because wMel is vulnerable to heat stress. wMel infections in their native host *Drosophila melanogaster* are genetically diverse, but few transinfections of wMel variants have been generated in *Ae. aegypti* mosquitoes. Here we successfully transferred a wMel variant (termed wMelM) originating from a field-collected *D. melanogaster* population from Victoria, Australia into *Ae. aegypti*. The new wMelM variant (clade I) is genetically distinct from the original wMel transinfection (clade III) generated over ten years ago, and there are no genomic differences between wMelM in its original and transinfected host. We compared wMelM with wMel in its effects on host fitness, temperature tolerance, *Wolbachia* density, vector competence, cytoplasmic incompatibility and maternal transmission under heat stress in a controlled background. wMelM showed a higher heat tolerance than wMel, with stronger cytoplasmic incompatibility and maternal transmission when eggs were exposed to heat stress, likely due to higher overall densities within the mosquito. Both wMel variants had minimal host fitness costs, complete cytoplasmic incompatibility and maternal transmission, and dengue virus blocking under standard laboratory conditions. Our results highlight phenotypic differences between closely related *Wolbachia* variants. wMelM shows potential as an alternative strain to wMel in dengue control programs in areas with strong seasonal temperature fluctuations.

Key words: *Aedes aegypti*, *Wolbachia*, microinjection, heat tolerance, wMel

Introduction

Aedes aegypti mosquitoes transmit some of the most important arboviral diseases such as dengue, which remain a major cause of morbidity and mortality across tropical regions (Kyle and Harris, 2008; Guzman *et al.*, 2010). A promising approach to reduce mosquito-borne disease involves the release of *Ae. aegypti* infected with the bacterium *Wolbachia* into wild populations (Hoffmann *et al.*, 2011;

Garcia *et al.*, 2016; Indriani *et al.*, 2020; Ahmad *et al.*, 2021; Wang *et al.*, 2021). *Wolbachia* are common intracellular bacteria that are transmitted maternally and have a range of effects on their insect hosts (Hoffmann and Turelli, 1997). *Wolbachia* often affect the reproduction of their hosts, particularly by causing cytoplasmic incompatibility (Hoffmann and Turelli, 1997), a phenomenon that results in sterility when a *Wolbachia*-infected male mates with an uninfected female or a female carrying a different infection (Caspari and Watson, 1959; Hoffmann and Turelli, 1997). Cytoplasmic incompatibility can be applied directly to suppress field mosquito populations through male-only releases (Zheng *et al.*, 2019; Crawford *et al.*, 2020; Beebe *et al.*, 2021; Ng and Consortium, 2021) or replace populations with mosquitoes carrying *Wolbachia* (Hoffmann *et al.*, 2011; Yen and Failloux, 2020). Replacement strategies are undertaken because *Wolbachia* reduce the transmission of dengue and other arboviruses by *Ae. aegypti* (Moreira *et al.*, 2009; van den Hurk *et al.*, 2012). Effective virus suppression is related to high *Wolbachia* infection frequencies in populations and high densities within individual mosquitoes (Lu *et al.*, 2012; Pinto *et al.*, 2021).

Through embryo microinjection, several *Wolbachia* infections including wMel, wMelPop, wMelCS and wAlbB have been established in *Ae. aegypti* following interspecific transfers from *Drosophila*, *Culex* and *Aedes* (Xi *et al.*, 2005; McMeniman *et al.*, 2009; Walker *et al.*, 2011; Fraser *et al.*, 2017). Releases to replace existing populations with those carrying *Wolbachia* have been achieved with both the wMel and wAlbB strains (Hoffmann *et al.*, 2011; Nazni *et al.*, 2019; Tantowijoyo *et al.*, 2020). wMel originates from *D. melanogaster* (Hoffmann, 1988; Walker *et al.*, 2011) and is now widely used for population replacement in *Ae. aegypti*, with releases undertaken in at least 10 countries including Australia, Brazil, Vietnam and Indonesia (Hoffmann *et al.*, 2011; Garcia *et al.*, 2019; Tantowijoyo *et al.*, 2020; Hien *et al.*, 2021). Mosquitoes with the wAlbB infection have been released in Malaysia, Australia, USA and Singapore for either population replacement or suppression (Nazni *et al.*, 2019; Staunton *et al.*, 2019; Crawford *et al.*, 2020; Beebe *et al.*, 2021; Ng and Consortium, 2021). There is now evidence of reduced dengue transmission after *Wolbachia* releases following replacement with both wMel and wAlbB (Nazni *et al.*, 2019; Ryan *et al.*, 2019; Indriani *et al.*, 2020; Utarini *et al.*, 2021).

The long-term success of *Wolbachia* population replacement strategies will depend on the stability of *Wolbachia* infections in populations and their phenotypic effects (Bull and Turelli, 2013; Hoffmann *et al.*, 2015; Ross *et al.*, 2019a). One of these factors is the ability to maintain *Wolbachia* effects under fluctuating temperatures. Compared to the range that their hosts can tolerate, *Wolbachia* infections may be more vulnerable to temperature extremes (Corbin *et al.*, 2017; Ross *et al.*, 2017). However, *Wolbachia* strains in *Ae. aegypti* respond differently to heat stress, with wAlbB-infected mosquitoes maintaining stronger cytoplasmic incompatibility, maternal transmission and *Wolbachia* density under heat stress compared to wMel (Ross *et al.*, 2017). In addition, the lack of selection response in wMel for increased heat resistance (Ross and Hoffmann, 2018) could limit the suitability of this strain in locations with extreme temperature fluctuations. Cold temperatures and long-term quiescence of eggs can also reduce *Wolbachia* densities, which may lead to reduced virus blockage or cytoplasmic incompatibility following a dry season (Lau *et al.*, 2020; Lau *et al.*, 2021).

The wMel strain is genetically diverse in natural *D. melanogaster* populations, with six major intraspecific clades (Richardson *et al.*, 2012; Early and Clark, 2013; Ilinsky, 2013). Patterns of wMel clades have shifted in recent decades, with the global spread of the wMel variant (clade III) replacing many wMel-CS-like (clade VI) variants (Riegler *et al.*, 2005). wMel variants have divergent phenotypic effects on hosts, inducing different levels of protection against viruses (Chrostek *et al.*, 2013), expression of cytoplasmic incompatibility (Veneti *et al.*, 2003) and fitness costs (Chrostek *et al.*, 2013). *Wolbachia* titres also vary across lines and populations which may relate to wMel variants (Chrostek *et al.*, 2013; Early and Clark, 2013). The incidence of these variants in *D. melanogaster* is also affected by environmental conditions, with lower *Wolbachia* infection frequencies in cooler temperate regions (Hoffmann, 1988; Kriesner *et al.*, 2016). Temperature influences the phenotypic effects of wMel,

including levels of virus protection (Chrostek *et al.*, 2021) and female fertility (Kriesner *et al.*, 2016). However, these different environmental responses may vary among wMel and wMel-like variants as indicated by impacts of variants on maternal transmission under cool temperatures (Hague *et al.*, 2022), host temperature preference (Truitt *et al.*, 2019; Hague *et al.*, 2020a) and survival following heat stress (Gruntenko *et al.*, 2017; Burdina *et al.*, 2021).

As wMel that has so far been transfected into *Ae. aegypti* is vulnerable to heat stress, and wMel variants in *D. melanogaster* have different thermal responses, we were motivated to generate new wMel variants in *Ae. aegypti* which could show greater environmental stability. Here we report the generation and characterization of an *Ae. aegypti* line (termed wMelM) infected with a wMel variant from *D. melanogaster* collected near Melbourne, Australia. We compared wMelM to the original wMel strain (generated by Walker *et al.*, 2011) in terms of life history, quiescent egg viability, *Wolbachia* density, vector competence and cytoplasmic incompatibility and maternal transmission under cold and heat stress. We found wMelM had higher heat tolerance without an obvious fitness cost while maintaining dengue blocking, providing a new strain that may be useful for release in different contexts.

Methods

Insect strains and colony maintenance

Aedes aegypti mosquitoes were reared in temperature-controlled insectaries at 26 ± 1 °C with a 12 h photoperiod following Ross *et al.* (2017). Larvae were reared in trays filled with 4L of reverse osmosis (RO) water and provided with fish food (Hikari tropical sinking wafers, Kyorin food, Himeji, Japan) *ad libitum* throughout their development. Four to five hundred adults were maintained in BugDorm-1 (27 L) cages (Megaview Science C., Ltd., Taichung, Taiwan). Four-to-six-day old female mosquitoes were starved for 24 hr, then fed on the forearm of a human volunteer. Blood feeding of female mosquitoes on human volunteers was approved by the University of Melbourne Human Ethics Committee (approval 0723847). All adult subjects provided informed written consent (no children were involved).

Three *Ae. aegypti* populations were used in this study, with an additional population (wAlbB) used in DENV vector competence experiments. wMel-infected mosquitoes were collected from Cairns, Australia in 2013 from areas where wMel had established in the wild population (Hoffmann *et al.*, 2011) following release of the wMel strain developed by Walker *et al.* (2011). The strain was developed with the shell vial technique which was used to infect the RML-12 cell line with wMel *Wolbachia* from *D. melanogaster* embryos from a laboratory stock (yw^{67C23}) and maintained by continuous serial passage. *Wolbachia* was then purified from this cell line and injected into *Ae. aegypti* embryos to transfer the wMel infection (Walker *et al.*, 2011). In contrast, the wMelM-infected mosquitoes in our study were generated by transferring cytoplasm directly from field-collected *D. melanogaster* through microinjection (see *embryonic microinjection* below). wMel.tet mosquitoes were cured of their wMel infection by treating adults with 2 mg/mL tetracycline hydrochloride in a 10% sucrose solution across two consecutive generations. wAlbB-infected mosquitoes were generated through microinjection as described previously (Ross *et al.*, 2021). Before experiments commenced, all populations were backcrossed to an uninfected population collected from Cairns, Australia for three consecutive generations to control for genetic background.

To generate a line for source material for microinjection for the current study, *D. melanogaster* were collected from the Yarra Valley, Victoria, Australia in April 2019. Isofemale lines were established and their progeny were screened for *Wolbachia* infection (see *Wolbachia* detection and density), with only

eggs from *Wolbachia*-infected lines used in experiments. Flies were maintained on cornmeal media 19 ± 1 °C with a 12 h photoperiod (Hoffmann *et al.*, 1986).

Embryonic microinjection

wMelM from *D. melanogaster* was introduced to *Ae. aegypti* through embryonic microinjection. Cytoplasm was removed from approximately 1 hr old donor eggs and injected directly into the recipient embryo according to Xi *et al.* (2005). Recipient eggs were collected by placing cups filled with larval rearing water and lined with filter paper (diameter 90 mm) into cages of mosquitoes blood fed >4 d prior. Eggs (<1.5 hr old, light gray in color) were lined up on filter paper and transferred to a cover slip with double-sided tape. Eggs were covered in 100% halocarbon oil and injected using a MINJ-1000 microinjection system (Tritech Research, Los Angeles, CA, USA). Eggs were gently removed from the oil after 10 minutes using a fine paintbrush and placed on a moist piece of filter paper. Eggs were hatched 3 d post-injection by submerging filter papers in containers filled with RO water, a few grains of yeast and half tablet of fish food. Hatching larvae were reared to adulthood and F₀ females were crossed to uninfected males, blood fed and isolated for oviposition. We screened the females for wMelM infection after producing viable offspring using LAMP (Goncalves *et al.*, 2019; Jasper *et al.*, 2019). This crossing process was repeated for three further generations, with only progeny from *Wolbachia*-positive females contributing to the next generation. We stopped the backcrossing and the detection once the wMelM infection reached fixation.

Whole genome sequencing

We sequenced the whole *Wolbachia* genome of the wMelM transinfection generated in this study at F₃ post-transinfection. We also sequenced populations from three lines at F₇, which were derived from three single F₁ females. Genomic DNA was extracted from a pooled sample of 5 individuals using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). Extracted DNA was randomly fragmented to a size of 350bp then end-polished, A-tailed, and ligated with Illumina sequencing adapters using a NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs, Ipswich MA, USA), and further PCR enriched with primers of P5 and P7 oligos. The PCR products which comprised the final libraries were purified (AMPure XP system, Beckman Coulter Life Sciences, Indianapolis IN, USA) and subjected to quality control tests that included size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA), and molarity measurement using real-time PCR. The libraries were then pooled and sequenced on a NovaSeq 6000 (Illumina) using 2 × 150 bp chemistry by Novogene HK Company Limited, Hong Kong.

Genome assembly

Quality filtering of raw sequencing reads was performed with Trimmomatic (Bolger *et al.*, 2014), using the following settings: leading = 20, trailing = 20, slidingwindow = 4:20, minlen = 70. Adapter sequences were removed using the ILLUMINACLIP function, with maximum seed mismatches = 2 and the palindrome clip threshold = 30. Filtered reads were mapped to a wMel reference genome (GenBank accession: NC_002978.6) and an *Ae. aegypti* mitochondrial reference genome (GenBank accession: NC_035159.1) using the Burrows-Wheeler Aligner (BWA), with the bwa mem algorithm and default parameters (Li, 2013). SAMtools and BCFtools were used to perform quality filtering of alignments and variant calling (Li *et al.*, 2009; Danecek *et al.*, 2021). PCR duplicates were excluded from downstream analyses by soft masking. Reads with a MAPQ score < 25 were removed from alignments, except for those with MAPQ = 0, which were retained to allow for mapping to repetitive regions. A maximum of 2000 reads per position were used to calculate genomic likelihoods. Ploidy was set to haploid for variant calling. The variant call output was used to create a consensus genome

sequence for each sample, wherein low coverage positions (depth < 5) were masked as 'N'. For *Wolbachia* genomes, Kraken2 (Wood *et al.*, 2019) was used to search for sequence contamination with the Standard-8 precompiled reference database (<https://benlangmead.github.io/aws-indexes/k2>; downloaded 17/9/21). The sequencing reads mapped by bwa to the wMel reference genome were filtered to remove reads matching taxa other than *Wolbachia*, and genome assemblies were then repeated with the filtered datasets, using the above pipeline. Genome sequences were inspected and aligned with Geneious v 9.1.8 (<https://www.geneious.com>). Read mapping densities were analysed with CNVpytor (Suvakov *et al.*, 2021) using the read depth approach, in order to search for putative deletions and duplications within the wMel genomes. Bin sizes of 100 and 500 were tested for the histogram, partition and CNV call steps.

Phylogenetic analysis

A phylogenetic tree was constructed using the wMel genomes from the present study and two large genome-wide SNP datasets that include representatives of the different wMel clades, obtained from *D. melanogaster* sampled from various international locations (Richardson *et al.*, 2012; Chrostek *et al.*, 2013; Early and Clark, 2013). Polymorphic sites that were common to both datasets were combined into a single SNP matrix, with sites that contained ambiguous base calls in any sample excluded from the analysis. A total of 58 polymorphic loci were retained. Maximum likelihood trees were constructed with RAxML-HPC v8.2.12 (Stamatakis, 2014) on XSEDE, using a GTR-GAMMA model with the Lewis method of ascertainment bias correction (Lewis, 2001), 44 alignment patterns and rapid bootstrapping (100 inferences). Bootstrap scores were plotted onto the best scoring ML tree. RAxML-HPC was accessed through the CIPRES Science Gateway (Miller *et al.*, 2010).

Life history parameters and cytoplasmic incompatibility

To compare the effects of wMelM and wMel infection on mosquito life history, we performed phenotypic assessments of the wMel, wMelM and wMel.tet populations. Fresh stored eggs (<1 week old) from each population were hatched in trays filled with 3L of RO water, a few grains of yeast, and one tablet of fish food. One day after hatching, 100 larvae were counted into trays filled with 500 mL of RO water and provided with fish food *ad libitum*, with 12 replicate trays per population. To determine the average larval development time for each tray, pupae were counted and sexed every day in the morning and evening. Pupae from each population were pooled across replicate trays (but with sexes separate) and used in the following experiments. The remaining pupae were pooled across sexes and released into BugDorm-1 cages for the quiescent egg viability experiment.

To determine adult longevity, 25 females and 25 males were placed in 3 L cages with cups of 10% sucrose and water, with eight replicate cages per population. Females were blood fed once per week. Dead mosquitoes were recorded and removed three times per week until all mosquitoes had died.

To determine female fertility and patterns of cytoplasmic incompatibility between wMel variants, we set up reciprocal crosses between wMel, wMelM and wMel.tet adults. Fifty females (1 d old) from the wMel, wMelM or wMel.tet populations were aspirated into 3 L cages containing 50 wMel, wMelM or wMel.tet males (1 d old), for a total of 9 crosses. Five d old females were blood fed and 30 engorged females per cross were isolated for oviposition in 70 mL specimen cups with mesh lids that were filled with 15 mL of larval rearing water and lined with a strip of sandpaper (Norton Master Painters P80 sandpaper, Saint-Gobain Abrasives Pty. Ltd., Thomastown, Victoria, Australia). Eggs were collected, partially dried, and then hatched three days after collection. Female fecundity was determined by counting the total number of eggs on each sandpaper strip, while hatch proportions were determined by dividing the number of hatched eggs (with a clearly detached egg cap) by the total number of eggs per female. Females that did not lay eggs or died before laying eggs were excluded from the analysis.

To evaluate fertility across gonotrophic cycles, females from the within-strain crosses (e.g. wMel x wMel) were blood fed again after laying eggs. Eggs were collected from four gonotrophic cycles in total, when females were 9, 14, 19 and 23 d old.

As an estimate of body size, one wing each from 20 males and 20 females per population was dissected and measured for its length (Ross *et al.*, 2016). Damaged wings excluded from the analysis.

Quiescent egg viability

To test quiescent egg viability which is an important ecological trait that can be affected by *Wolbachia* infections (Lau *et al.*, 2021), cages of 5 d old females from wMel, wMelM or wMel.tet populations were blood fed and six cups filled with larval rearing water and lined with sandpaper strips were placed inside in each cage. Eggs were collected five days after blood feeding, partially dried, then placed in a sealed chamber with an open container of saturated potassium chloride (KCl) solution to maintain a constant humidity of ~84%. When eggs were 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 weeks old, small sections of each sandpaper strip were removed and submerged in water with a few grains of yeast and fish food to hatch. Twelve replicate batches of eggs were hatched per population at each time point, with around 50-100 eggs per batch. Hatch proportions were determined by dividing the number of hatched eggs (with a clearly detached egg cap) by the total number of eggs in each batch.

Wolbachia density across life stages

We compared the *Wolbachia* density of wMelM and wMel during development by storing random subsets of 1st instar larvae, 3rd instar larvae, female pupae, male pupae, female adults and male adults (within 24 hr of emergence) in 100% ethanol. Fifteen individuals from each life stage, sex and wMel variant were then measured for *Wolbachia* density using qPCR (see “*Wolbachia* detection and density”).

DENV-2 virus challenge and quantification

We measured the vector competence of wMelM for DENV-2 relative to two other *Wolbachia* variants (wMel and wAlbB) and a matched uninfected population (wMel.tet). Experiments were performed in a quarantine insectary with *Ae. aegypti* held in incubators (PG50 Plant Growth Chambers, Labec Laboratory Equipment, Marrickville, NSW, Australia) set to a constant 26°C with a 12:12 light: dark photoperiod. Eggs (10 d old) from the wMel, wMelM, wMel.tet and wAlbB populations were hatched and larvae were reared to adulthood in five replicate trays of 100 larvae per temperature treatment for each population. Adults were released into 13.8-liter cages (BugDorm-4F2222, Megaview Science C Ltd., Taichung, Taiwan) and provided with 10% sucrose solution. Adults were starved for 24 hr before virus challenge and experiments were performed with 6-7d old females.

We performed virus challenge experiments with a 1×10^7 TCID₅₀/mL dose of DENV-2 in human blood. DENV-2 (Cosmopolitan) provided by VDRL and isolated in Melbourne from a traveler in 2016 and was grown in C6/36 cells before use in experiments. Females were fed human blood sourced from the Red Cross under agreement number 16-10VIC-02 and spiked with DENV-2. Blood was provided through 6 mL Hemotek membrane feeders (Hemotek Ltd., Blackburn Lancashire, Great Britain) which were placed on the top of each cage for 30 min and heated with pocket hand warmers (Kathmandu). Engorged mosquitoes were transferred to cages with sucrose solution and cups lined with sandpaper strips for oviposition, with non-blood fed mosquitoes discarded. Females were maintained at 26°C for 12 d before processing. Cages were chilled briefly, then heads were dissected from bodies on a cold plate and stored individually in 1.7 mL tubes with 100 µL crushing media (DMEM with 1000u/ml

streptomycin/penicillin and 2µg/ml amphotericin B) and two 3mm glass beads. Tubes were stored at -20°C for 48 hr before removal from the quarantine insectary for long-term storage at -80°C.

Virus titres were quantified from whole bodies (heads removed) from 30 individuals per population with TCID₅₀ assays as described previously (Duchemin *et al.*, 2017). Briefly, 30 µl of crushed mosquito were serially 10-fold diluted in medium (DMEM containing 2% FBS). Using 96-well plates, 50 µl of each dilution was sequentially placed in wells (6 replicates). 100µl of fresh medium containing Vero cells (final cell confluency of 50-60%) was then overlaid. The cells were incubated for 7 days before they were observed for cytopathic effect (CPE). The 50% endpoint was calculated using the Reed and Muench method (Reed and Muench, 1938). The infection rate was calculated as the proportion (percentage) of all experimentally infected mosquitoes (n = 30) in which DENV was detected.

Cytoplasmic incompatibility and *Wolbachia* density following cold and heat stress

We measured cytoplasmic incompatibility and *Wolbachia* density in adults after being exposed to cyclical cold or heat stress during the egg stage for one week. Eggs were collected on sandpaper from both *Wolbachia*-infected populations. Four days after collection, batches of 40-60 eggs were brushed off the sandpaper and tipped into 0.2 mL PCR tubes (12 replicate tubes per population) and exposed to cyclical temperatures of 3-13°C, 4-14°C, 6-16°C, 27-37°C, 28-38°C or 29-39°C for 7 d in Biometra TProfessional TRIO 48 thermocyclers (Biometra, Göttingen, Germany) according to Kong *et al.* (2016) and Ross *et al.* (2019b). Eggs of the same age from each population, as well as wMel.tet eggs, were kept at 26°C. Eggs from all treatments were brought to 26°C and hatched synchronously. Larvae were reared at a controlled density (up to 100 larvae per tray of 500 mL water). Pupae were sexed and 8-30 adults per population were stored in absolute ethanol within 24 hr of emergence for *Wolbachia* density measurements (see *Wolbachia* detection and density). The remaining pupae were left to emerge into 3 L cages (with each sex, temperature treatment and population held in separate cages) for cytoplasmic incompatibility crosses. Due to low survivorship of eggs held at 3-13°C and 4-14°C, individuals from these treatments were not used in crossing experiments.

We established two sets of crosses to test cytoplasmic incompatibility induced by *Wolbachia*-infected males and test the ability of *Wolbachia*-infected females to restore compatibility with *Wolbachia*-infected males. In the first set, untreated wMel.tet females were crossed with *Wolbachia*-infected males from each temperature treatment. In the second set, *Wolbachia*-infected females from each temperature treatment were crossed with untreated *Wolbachia*-infected males. Five-day old females were blood-fed and 20 females per cross were isolated for oviposition. Hatch proportions per female were determined according to previous experiments (see above).

Maternal transmission of *Wolbachia*

We compared the ability of wMel and wMelM females to transmit *Wolbachia* infections to their offspring after parental eggs were exposed to cyclical heat stress (28-38°C) for one week, or held at 26°C. Eggs were returned to 26°C and reared to adulthood, and wMel and wMelM females from each temperature treatment were crossed to wMel.tet males. Twenty females per cross were isolated for oviposition after blood feeding. We scored 10 offspring for their *Wolbachia* infection status from 10 females per infection type at each temperature to assess the loss of *Wolbachia* in next generation.

***Wolbachia* detection and density**

We used LAMP assays for rapid detection of wMel during microinjection experiments according to Jasper *et al.* (2019) using wMel-specific primer sets (Goncalves *et al.*, 2019). qPCR assays were used

to confirm the presence or absence of *Wolbachia* infection and measure relative density in all other experiments (Lee *et al.*, 2012; Axford *et al.*, 2016). DNA was extracted using 250 µL of 5% Chelex 100 resin (Bio-Rad laboratories, Hercules, CA) according to methods described previously (Hoffmann *et al.*, 2014). used a LightCycler® 480 High Resolution Melting Master (HRMM) kit (Roche; Cat. No. 04909631001, Roche Diagnostics Australia Pty. Ltd., Castle Hill New South Wales, Australia) and IMMOLASE™ DNA polymerase (5 U/µl) (Bioline; Cat. No. BIO-21047) as described by Lee *et al.* (2012). Three primer sets were used to amplify markers specific to mosquitoes (mRpS6_F 5'AGTTGAACGTATCGTTTCCCGCTAC3' and mRpS6_R 5' GAAGTGACGCAGCTTGTGGTCGTCC3'), *Ae. aegypti* (aRpS6_F 5'ATCAAGAAGCGCCGTGTCG3' and aRpS6_R 5'CAGGTGCAGGATCTTCATGTATTTCG3'), and wMel (w1_F 5'AAAATCTTTGTGAAGAGGTGATCTGC3' and w1_R 5' GCACTGGGATGACAGGAAAAGG3'). Relative *Wolbachia* densities were determined by subtracting the Cp value of the *Wolbachia*-specific marker from the Cp value of the mosquito-specific marker. Differences in Cp were averaged across 2-3 consistent replicate runs, then transformed by 2ⁿ.

Statistical analysis

All analyses were conducted using SPSS statistics version 26.0 for Mac (SPSS Inc, Chicago, IL). Egg hatch proportions were characterized per female (i.e. individual eggs were not treated as independent data points) and this trait as well as longevity were not normally distributed according to Shapiro-Wilk tests, therefore we analyzed these data with nonparametric log-rank tests for longevity and Kruskal-Wallis for egg hatch proportions. The development time for females and males, fecundity, *Wolbachia* density under heat and cold were analyzed by two-way ANOVAs. *Wolbachia* density for different life stages was compared with a t test. DENV-2 TCID₅₀ comparisons across strains were undertaken using a Mann-Whitney test and differences in the infection rate were evaluated using two-tailed Fisher's tests.

Results

Genomic analyses of the wMelM transinfection

The genome of the original wMel transinfection (Walker *et al.*, 2011) was very similar to both the wMel reference genome (Wu *et al.*, 2004) and the wMel genome reported by Chrostek *et al.* (2013), differing from each by five and four polymorphic loci respectively, consistent with the three strains having originated from same *Drosophila* stock (*yw*^{67C23}) less than 5,000 generations ago (Table S2). Phylogenetic analysis placed these genomes in a single monophyletic cluster together with the other members of wMel clade III (Figure 1).

Clear differences were observed between the genome sequence of the original wMel transinfection strain and the wMelM variant. Phylogenetic analysis placed wMelM within wMel clade I, with greatest similarity to wMel variants from Ithaca NY, USA and Tasmania, Australia (Figure 1). Thirty-six SNPs and small indels were identified, with approximately half predicted to cause a change to an amino acid sequence (Table S2). Several of these changes were located within proteins with functions including transportation and secretion; amino acid, porphyrin, and nucleotide metabolism; and DNA replication and repair, while many others were located within hypothetical proteins of unknown function. Most SNPs identified were the same SNPs identified by Hague *et al.* (2022) in their comparison of wMel genomes from temperate (clade III) and tropical (clade I) *D. melanogaster*, with five of these being significantly associated with temperature according to their analysis ($P < 0.05$ see Hague *et al.*, 2022). Analysis of read mapping densities, performed with CNVpytor, did not identify any regions of significant copy number variation within the wMelM genome, when compared to the wMel reference genome. We did, however, identify two large deletions through standard genome assembly – one within a gene encoding a S49 family peptidase and one within an ankyrin repeat containing gene.

Examination of allele frequencies indicated that in addition to *wMelM*, a *wMel* variant with high identity to the *wMel* reference sequence is likely to have been present within the *Drosophila wMelM* donor population, but only *wMelM* appears to have been retained within the transinfected mosquito populations. The *wMelM* genome sequences in *Ae. aegypti* at F₃ and three isofemale lines at F₇ were identical, suggesting no genetic changes across several generations following transinfection. Relative to the *Ae. aegypti wMelM* genomes, the *Drosophila wMelM* consensus sequence contained eight SNPs and lacked the two large deletions mentioned above. It is probable that these differences are artifacts introduced by the presence of the other *wMel* variant in this sample.

The mitochondrial genome sequences of the five mosquito populations included in the study were very similar, with only five SNPs observed between them, four of which were located outside of known coding regions. Most of the SNPs were associated with anomalously low read depths, relative to their surrounding positions, making it likely that they represent sequencing artifacts.

Figure 1: Genome-wide SNP phylogenetic analysis of *wMel* variants. SNP data from the six genomes in this study and 253 previously published *wMel* genomes were analysed (Richardson *et al.*, 2012; Chrostek *et al.*, 2013; Early and Clark, 2013). The *wMel* variant highlighted in red is the original *wMel* transinfection (Walker *et al.*, 2011) used in phenotypic comparisons with *wMelM*. *wMelM* sequences are highlighted in blue. Maximum likelihood trees were constructed with RAxML-HPC using 58 SNP loci and ascertainment bias correction; scale bar = number of substitutions per SNP matrix site. Nodes with bootstrap values less than 50% have been collapsed; triangle height = length of longest branch within node. *Wolbachia wMel* clades are shown on the right. In cases where multiple samples had identical SNP haplotypes, one representative sequence was used for tree construction. The number of sequences corresponding to each entry is shown in parentheses. For wild populations, the location of sampling is shown (refer to Table S1 for sample names). AUS = Australia; CHN = China; CMR = Cameroon; COD = Democratic Republic of the Congo; ETH = Ethiopia; FRA = France; GAB = Gabon; GIN

= Guinea; KEN = Kenya; NGA = Nigeria; NLD = Netherlands; RWA = Rwanda; TZA = Tanzania; UGA = Uganda; USA = USA; ZAF = South Africa; ZMB = Zambia; ZWE = Zimbabwe; DGRP = Drosophila Genetic Reference Panel (originally sampled from Raleigh NC, USA).

Limited effects of wMelM on life history traits

We compared the effects of wMel and wMelM infection on *Ae. aegypti* life history traits. Overall, wMelM infection had no effect, or slightly increased mosquito fitness compared to uninfected (wMel.tet) mosquitoes (Figure 2). *Wolbachia* infection had no clear effect on development time (two-way ANOVA: $P = 0.124$ for females and 0.194 for males, Figure 2A and 2B). Both wMel variants increased adult female longevity (Log-rank test: wMel vs wMel.tet, $P = 0.011$, $\chi^2 = 6.436$, $df = 1$, wMelM vs wMel.tet: $P = 0.019$, $\chi^2 = 5.501$, $df = 1$, Figure 2C) relative to wMel.tet, but male longevity was unaffected by *Wolbachia* infection status (Log-rank test: wMel vs wMel.tet, $P = 0.251$, $\chi^2 = 1.32$, $df = 1$, wMelM vs wMel.tet: $P = 0.091$, $\chi^2 = 2.849$, $df = 1$, Figure 2D). Fecundity was influenced by *Wolbachia* infection type (two-way ANOVA: $F_{2,326} = 3.753$, $P = 0.024$), with wMelM females consistently laying more eggs than the wMel and wMel.tet females (Figure 2E). Median egg hatch proportions were close to 100% across all gonotrophic cycles, regardless of *Wolbachia* infection type (Figure 2F). wMel males had smaller wings than wMel.tet males (one-way ANOVA: $P = 0.04$, Figure 1H) with no difference between the two wMel variants ($P = 0.767$), while female wing length was unaffected by *Wolbachia* infection type ($P > 0.05$, Figure 2G).

Figure 2. Life history parameters of backcrossed wMel, wMelM and wMel.tet *Aedes aegypti* populations. The wMel (blue), wMelM (red) and wMel.tet (green) populations were evaluated for the following traits: larval development time for (A) females and (B) males, longevity of (C) females and (D) males, (E) female fecundity across gonotrophic cycles, (F) egg hatch proportion across gonotrophic

cycles, and wing length of (G) females and (H) males. Each point represents data averaged across a replicate container of 100 individuals (A-B) or data from individual mosquitoes (panels G-H). Medians and 95% confidence intervals are shown in lines and error bars.

wMel and wMelM induce complete cytoplasmic incompatibility

We tested the ability of wMel and wMelM-infected males to induce cytoplasmic incompatibility with wMel.tet females. These females produced no viable offspring when crossed to wMel and wMelM males, indicating that both variants induce complete cytoplasmic incompatibility (Table 1). Reciprocal crosses between wMel and wMelM variants resulted in high egg hatch proportions (>90%), indicating that the two variants are bidirectionally compatible (Table 1).

Table 1. Cytoplasmic incompatibility between wMel, wMelM and wMel.tet *Aedes aegypti* populations.

		Female		
		wMel.tet	wMel	wMelM
Male	wMel.tet	0.980 (0.967, 1)	0.930 (0.867, 0.969)	0.952 (0.904, 0.990)
	wMel	0 (0, 0)	0.971 (0.945, 0.983)	0.981 (0.955, 0.990)
	wMelM	0 (0, 0)	0.963 (0.850, 0.986)	0.962 (0.942, 0.986)

Median egg hatch proportions are shown with 95% confidence intervals (lower, upper).

wMel variants decrease quiescent egg viability

Stored eggs from each population were hatched every two weeks to determine quiescent egg viability. Egg viability decreased across time for all three infection types, but hatch proportions for both wMel variants were lower than wMel.tet from week 2 onwards (Kruskal-Wallis test, week 2: $H = 12.434$, $df = 2$, $P = 0.002$, Figure 3). wMel and wMelM did not differ significantly in hatch proportion across all weeks combined (statistical test $P = 0.114$), suggesting that the two variants have similar quiescent egg viability.

Figure 3. Quiescent egg viability of backcrossed wMel, wMelM and wMel.tet *Aedes aegypti* populations across 22 weeks. Symbols show median egg hatch proportions while error bars show 95% confidence intervals.

wMel variants have similar *Wolbachia* density across life stages

Wolbachia density in whole individuals differed across life stages (two-way ANOVA: $F_{5,177} = 75.85$, $P < 0.001$), with low densities observed in third instar larvae (Figure 4). We did not find differences in pairwise comparisons between the *wMel* and *wMelM* strains (t-test: all $P > 0.05$) except for male pupae ($P = 0.011$), where *wMelM* male pupae had higher *Wolbachia* densities than *wMel* male pupae (Figure 4). Overall, the *Wolbachia* density of *wMelM* was slightly higher than *wMel* across all life stages but this effect was not significant (two-way ANOVA: $F_{1,177} = 2.636$, $P = 0.106$).

Figure 4. *Wolbachia* density of different life stages in backcrossed *wMel* and *wMelM* *Aedes aegypti* populations. Each point represents the relative density for an individual averaged across 2-3 technical replicates. Medians and 95% confidence intervals are shown in lines and error bars. “F” stands for females and “M” stands for males.

***wMelM* blocks DENV-2**

We assessed the vector competence of *wMelM*-infected *Ae. aegypti* by feeding mosquitoes an infectious blood meal and comparing DENV-2 titres against *wMel*, *wAlbB* and *wMel.tet* populations. DENV-2 titres ($P = 0.016$) and the proportion of DENV-2 infected females ($P=0.033$) were significantly lower in *wMelM* compared to *wMel.tet* (Figure 5). There was no detectable difference between *wMelM* blockage and that of the other two *Wolbachia* strains, *wMel* and *wAlbB*, as measured by both DENV-2 replication and infection proportion (all $P > 0.05$).

Figure 5. wMelM inhibits DENV-2 replication in virus-fed *Ae. aegypti*. Statistical comparisons of DENV-2 titre were compared using a Mann-Whitney test where * indicates $P < 0.05$. Each point on the plot represents an individual infected mosquito (with negative mosquitoes excluded), and lines and error bars denote medians $\pm$ 95% CIs. The proportion of DENV-2 positive mosquitoes out of the total tested (proportion infected) is indicated below each plot.

wMelM induces stronger cytoplasmic incompatibility than wMel under cyclical heat stress

We tested the ability of wMel and wMelM-infected males to induce cytoplasmic incompatibility when eggs were exposed to cold or heat stress. Males from both wMel and wMelM lines induced complete cytoplasmic incompatibility with wMel.tet females at 6-16°C and 26°C (Figure 6A). Incomplete cytoplasmic incompatibility was observed at 27-37°C and above, with an increasing proportion of viable offspring produced as the temperature increased (Figure 6A). However, wMelM retained stronger cytoplasmic incompatibility than wMel at high temperatures, with significantly lower egg hatch proportions when males were exposed to 28-38°C (Kruskal-Wallis test: $H = 6.743$, $df = 1$, $P = 0.009$) and 29-39°C ($H = 5.344$, $df = 1$, $P = 0.021$).

Wolbachia-infected females exposed to cyclical temperatures as eggs were crossed to *Wolbachia*-infected males reared at 26°C to test the ability of females to restore compatibility (Figure 6B). Compared to wMelM, wMel had a slightly decreased hatch rate under cold stress but treatments did not differ significantly ($H = 1.838$, $df = 1$, $P = 0.175$). After the exposure to cyclical temperatures of 27-37°C for 7 d, hatch rate reduced. Hatch rates in both strains decreased with temperature, but wMelM had a higher hatch rate under cyclical temperatures of 28-38°C (Kruskal-Wallis test: $H = 18.000$, $df = 1$, $P < 0.001$) and 29-39°C ($H = 13.766$, $df = 1$, $P < 0.001$).

Figure 6. Cytoplasmic incompatibility induction and compatibility restoration by wMel/wMelM variants under cold and heat stress. We performed crosses to test the ability of (A) *Wolbachia*-infected males to induce cytoplasmic incompatibility with wMel.tet females and (B) *Wolbachia*-infected females to restore compatibility with *Wolbachia*-infected males. We also evaluated egg hatch proportions from crosses between (C) *Wolbachia*-infected females and wMel.tet males used in the maternal transmission experiment. In each cross, males (A) or females (B, C) were exposed to cyclical temperatures for 7 d or held at 26°C during the egg stage. Each point represents the hatch proportion of eggs from a single female. Bars show medians while error bars show 95% confidence intervals.

wMelM has increased maternal transmission fidelity compared to wMel under cyclical heat stress

We compared the ability of wMel and wMelM females to transmit *Wolbachia* to their offspring when they were exposed to 28-38°C as eggs for 7d or reared at a constant 26°C (Figure 6C). wMel and wMelM females transmitted the infection to all their offspring at 26°C. In contrast, wMel and wMelM-infected females failed to transmit the infection to some of their progeny at 28-38°C (Figure 7). More progeny from wMelM mothers had *Wolbachia* than from wMel mothers ($66.7\% \pm 6.87\%$) at a cycling 28-38°C (Kruskal-Wallis test: $H=3.891$, $df=1$, $P=0.048$, Figure 7), suggesting that the transmission of wMelM may be more stable at high temperatures.

Figure 7. Maternal transmission of wMel/wMelM variants following maternal heat stress exposure. *Wolbachia*-infected females were exposed to cyclical temperatures of 28-38°C for 7 d or held at 26°C during the egg stage, then crossed to wMel.tet males. Each point represents the percent of *Wolbachia*-positive offspring from a single female. Medians and 95% confidence intervals are shown in lines.

wMelM has a higher density than wMel under heat and cold stress

We measured *Wolbachia* density when eggs were exposed to cold or heat stress. We found no significant effect of sex on *Wolbachia* density ($P > 0.05$), so data from males and females were pooled for the following analysis. In the two-way ANOVA, *Wolbachia* density was influenced both by the wMel variant ($F_{1,142} = 26.425$, $P < 0.001$) and temperature ($F_{2,142} = 247.264$, $P < 0.001$). At high temperatures, the density of wMel and wMelM decreased sharply (Figure 8B). For the lower temperature, the *Wolbachia* density was relatively stable but was still influenced by wMel variant ($F_{1,99} = 12.658$, $P = 0.001$) and temperature ($F_{2,99} = 18.323$, $P < 0.001$) (Figure 8A). Although wMel and wMelM had similar density at the adult stage when reared at constant 26°C (Figure 4), we did find that wMelM had a higher *Wolbachia* density under all the cycling heat and cold stress regimes (Figure 8). Compared to wMel, the density of wMelM was around 2 times higher overall in this experiment.

Figure 8. *Wolbachia* density in adult mosquitoes following exposure to cyclical (A) cold and (B) heat stress during the egg stage. Eggs were exposed to cyclical temperatures for 7 d. Each point represents the relative density for an individual averaged across 2-3 technical replicates. Medians are shown as short horizontal lines and lines join medians across different temperature cycles.

Discussion

We successfully used microinjection to transfer *Wolbachia* directly from *D. melanogaster* to *Ae. aegypti* to generate the wMelM line. wMelM differs from the original wMel transinfection (Walker *et al.*, 2011) both in terms of the source of the wMel material (laboratory versus field-derived *D. melanogaster*) and initial passage through a cell line. Although different methods were used to develop the wMel and wMelM transinfection, the wMelM variant like the original wMel has minimal effects on mosquito fitness. The wMelM variant was associated with a somewhat higher host fecundity compared to the wMel strain and a longer host life span when compared to wMel.tet mosquitoes, traits that would be likely to improve the success of release strategies in a field setting aimed at replacement. Apart from these fitness effects, we found minor differences in the density of *Wolbachia* which may be correlated with the degree of virus blocking (Lu *et al.*, 2012; Osborne *et al.*, 2012; Fraser *et al.*, 2017). For wMelM, *Wolbachia* density was higher but only under cycling heat and cold conditions; this leads to the expectation that virus blockage should be similar in the two strains, consistent with the similar virus blocking ability for wMelM, wMel and wAlbB observed at 26°C. The high level of transmission and incompatibility generated by wMelM under the varied thermal conditions tested here may facilitate its invasion into natural field populations and persistence at a high level.

In native *Drosophila* hosts, *wMel* *Wolbachia* density can vary substantially (Early and Clark, 2013); for instance, individuals from Ithaca NY, USA, most of which were infected with *wMel* from clade I, had higher *Wolbachia* titres when reared at room temperature than individuals from Tasmania, Australia, Zimbabwe and the Netherlands, most of which were infected with *wMel* from clade III. It is possible that a common mechanism underlies this finding and our observation of higher densities of *wMelM* (clade I) in temperature-stressed *Ae. aegypti* relative to the original *wMel* transinfection strain (clade III). However, the cause of these differences in *Wolbachia* density remain to be determined. In more distantly related *wMelCS*-like variants (clade VI), high *Wolbachia* titres are associated with both the loss of and the amplification of the Octomom genome region (Chrostek *et al.*, 2013; Chrostek and Teixeira, 2015; Duarte *et al.*, 2021). In our case, we did not detect any alteration of Octomom copy number in *wMelM*. However, we did observe non-silent changes in genes encoding a *Wolbachia* surface protein (*wspB*), a member of the type IV secretion system, and proteins involved in amino acid metabolism, all functions that are likely to be important in interactions between endosymbiont and host (Pichon *et al.*, 2009; Caragata *et al.*, 2014; Rice *et al.*, 2017; Jimenez *et al.*, 2019; Epis *et al.*, 2020).

Previous studies have shown that strong geographic structuring exists among global *wMel* populations. While multiple *wMel* variants can often be found within a single location or region, usually one variant predominates at any given location, suggestive of adaptation to local environmental conditions (Richardson *et al.*, 2012; Early and Clark, 2013). There have been very few changes in the original *wMel* strain since transinfection (Huang *et al.*, 2020) but this strain is different genomically from some *wMel* strains present in *D. melanogaster* from Australia (which provided the donor for *wMelM*). Phylogenetic analysis placed the original *wMel* transinfection (Walker *et al.*, 2011) within clade III, which contains 13 of the 14 other Australian (Tasmanian) samples included in the tree, and *wMelM* within clade I, which contains a single Tasmanian sample. This pattern raises the possibility that clade III *wMel* variants may have a competitive advantage over clade I variants in some parts of southern Australia, such as Tasmania, which lies at the southern boundary of the cline of decreasing *Wolbachia* infection incidence (Kriesner *et al.*, 2016). Most of the SNPs in the *wMelM* genome were identified in a study of *wMel* SNPs that distinguished different geographic climate zones, with five of these shown to be significantly associated with temperature (Hague *et al.*, 2022). In our analysis, we did find a SNP in the gene encoding the *Wolbachia* outer membrane protein B (*wspB*, WD0009), which results in a premature stop codon and is likely a major determinant of *Wolbachia* thermal sensitivity (Hague *et al.*, 2022). It may be that the polymorphisms in the *wMelM* genome and the higher densities observed for *wMelM* under heat and cold stress are selected against in regions with lower yearly minimum temperatures (Kriesner *et al.*, 2016). It would be interesting to determine the frequency of different *wMel* variants in natural *D. melanogaster* populations across Australia, especially in more northerly regions where *Ae. aegypti* population replacement programs are being conducted.

Phenotypic differences between *wMel* and *wMelM* may relate to the donor line of natural *Drosophila* used in the transinfection as well as the difference in methodology related to mosquito cell passaging rather than direct transfer. In eastern Australian *D. melanogaster*, *Wolbachia* were first identified from incompatibility generated through crossing experiments between populations from along a latitudinal cline (Hoffmann, 1988) and *wMel* shows a stable latitudinal pattern from being near 100% in incidence to being at a very low frequency (Kriesner *et al.*, 2016). This pattern suggests environmental effects on *Wolbachia* dynamics and/or differences in the *Wolbachia* strain/host backgrounds affecting these dynamics. Transplant experiments followed by semi-field cage experiments point to a complex pattern of *Wolbachia* and host associated fitness effects at ends of the cline (Olsen *et al.*, 2001) while experimental studies implicate environmental effects interacting with *Wolbachia* fitness (Kriesner *et al.*, 2016). Other data also point to different *wMel* variants changing over time at different temperatures (Versace *et al.*, 2014). Furthermore, environmental and genetic difference sometimes contribute together to *Wolbachia* transmission and frequency variation expected to influence the *Wolbachia* spread (Hague *et al.*, 2020b).

For *Wolbachia* infections generally, high temperatures can have an impact on release success by weakening the reproductive effects induced by *Wolbachia* (Trpis *et al.*, 1981; Johanowicz and Hoy, 1998; Ross *et al.*, 2017) and *Wolbachia* can even be eliminated under sufficient thermal stress (Trpis *et al.*, 1981; Stouthamer *et al.*, 1990). In the case of *Ae. aegypti*, high temperature effects depend on the *Wolbachia* strain, with wAlbB performing better than wMel (Ross *et al.*, 2017). The higher *Wolbachia* density of wAlbB under heat may explain the fact that its phenotypic effects are not altered much by high temperatures. This also appears to be the case for the wMelM variant.

Given the impact of high temperature on the dynamics of *Wolbachia*-infected mosquitoes and the benefits of *Wolbachia* releases targeting *Ae. aegypti* in several countries from different climate zones where dengue is common (Bhatt *et al.*, 2013; Ritchie, 2018), it may be prudent to consider multiple strains in releases in different environments (Ross *et al.*, 2019a). The wMelM variant generated here has minimal fitness effects but a higher phenotypic stability at high temperatures compared to wMel. Our results suggest that this new transinfected strain may be useful in environments where temperatures are variable. Given that temperature conditions also change seasonally, the new strain may allow for releases in spring when temperatures are often variable. It is also worth considering other strains from field populations for transfections.

Acknowledgements

We thank Kelly Richardson for collecting the *Drosophila* line used for microinjection, Marianne P. Coquilleau for the assistance in the experiments and Moshe Jasper for support with LAMP assays.

Funding

AAH was supported by the National Health and Medical Research Council (1132412, 1118640, www.nhmrc.gov.au). PAR was supported by a University of Melbourne Early Career Research Grant. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data and strain availability

The wMelM *Aedes aegypti* strain is available from the authors on request.

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