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Thermal stress during larval development reduces viral transmission blocking in *Wolbachia*-infected *Aedes aegypti*

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Abstract

Arboviral disease transmission has been attenuated using *Wolbachia*-infected *Aedes aegypti* in a variety of countries worldwide. However, recent findings suggest that the *w*Mel strain has limitations due to ecological challenges, which can dramatically affect its cellular density and thus its viral blocking capability. Hence, there are on-going studies that seek to find alternative and potentially superior *Wolbachia* strains that combine thermal stability with strong pathogen inhibition. Previous studies examining the effects of temperature on *Wolbachia* density have used basic and extreme temperature stress regimes (simply cycling between 27-37°C). Here we studied the effects of varied temperature patterns using field collected temperature data that more accurately reflects the natural temperature cycles in the field. We used two transinfected lines of *Aedes aegypti* carrying the *w*Mel and *w*AlbB *Wolbachia* strains. Using qPCR on whole-body and tissue dissections across *w*Mel, *w*AlbB and wild-type strains, there was a five-fold decrease in *Wolbachia* density in *w*Mel and no significant reduction in *w*AlbB across control and heat-treated strains. In the control (non-heat treated) *Wolbachia* infected lines; ovaries were the key reservoirs of *Wolbachia* across all tissues with low amounts being detected in midguts and salivary glands. Fluorescent *in situ* hybridisation on ovaries between control and heat-treated groups showed a reduction in the amount of signal in *w*Mel as compared to no reduction in *w*AlbB. The fidelity of maternal transmission was reduced in heat-treated *w*Mel, while *w*AlbB remained constant. Viral blocking capacity was affected after two generations of heat-treatment in *w*Mel lines, whereas *w*AlbB maintained strong viral blocking despite the high temperatures. These results clearly show that natural diurnal temperature fluctuations can have an adverse effect on *Wolbachia*-mediated viral suppression by reducing the levels of the endosymbiont

within the vector host. Considering temperature stability is therefore a key parameter in the selection of *Wolbachia* strains in the field studies against arboviral infections.

Author Summary

Currently, a worldwide threat exists from infectious arboviral diseases transmitted by *Aedes* mosquitoes. Different control methods have been established to limit this burden, however due to overwhelming challenges a definitive answer is still required. Recently, the heritable and endosymbiotic bacterium *Wolbachia* has proven promising because of its high capacity of population invasion and viral blocking capacity. This has led to candidate strains (such as *w*Mel—native *Wolbachia* in fruit flies) to be used in field controls of dengue and Zika in endemic areas. However, recent discoveries suggest that this strain suffers from thermal stress, which may limit its viral blocking and population invasion capacity. Here we emulated field collected temperature data from larval breeding areas and discovered that in addition to the previously reported tendency of *w*Mel density decline and experience maternal leakage, there is also a drop in viral blocking ability in *w*Mel as compared to the robust blocking by *w*AlbB after two generations of heat-treatment. Therefore, our findings show that for efficient and effective selection of *Wolbachia* strains for viral obstruction in the natural setting; heat stability should be a crucial factor to be taken into consideration.

Introduction

Arthropod borne viruses affect millions of people annually with high rates of morbidity and mortality resulting in an enormous health burden[1,2]. Dengue, which puts nearly half of the human population at risk of infection, lacks an effective vaccine. The recently emerged Zika virus also has no vaccine[3,4].The primary vectors for these viral diseases are *Aedes aegypti* and *Aedes albopictus*, of which the former infests urban settings while the latter is both an urban and peri-urban mosquito[5,6].

Various disease control strategies are in use ranging from fogging with insecticides to the use of indoor residual sprays, which are faced with resurgent resistance and a limited duration of effectiveness[7-9]. The obvious migration of humans into the urban settlements, together with the massive coverage that is required for the above-mentioned strategies to be effective, have made it extremely difficult to control these diseases[10,11].

Microbial interactions with mosquito hosts have been investigated in the context of blocking arboviral transmission. For example, *Wolbachia,* a maternally-transmitted endosymbiotic bacterium[12,13] that can induce reproductive manipulations such as cytoplasmic incompatibility (CI), male killing, haploid induced parthenogenesis and feminization[14], to promote its spread. These effects are often strain specific and result in different host fitness implications. *Wolbachia* can also directly influence vectorial competence through shortening of the mosquito lifespan and causing pathogen transmission blocking[15,16].

Wolbachia-mediated antiviral protection takes place for a wide range of RNA viruses (Zika, dengue, Chikungunya, Semiliki Forest Virus and several others) both in *Drosophila* and mosquitoes[17]. Certain parameters have been explored to quantify

this protection such as measuring number of infected virus individuals and viral load in whole or parts of mosquitoes, measuring dissemination and viral transmission via the salivary glands. The outcome has been either a modest infection rate or viral accumulation within infected hosts to near clearance of the viral replication and transmission. These studies have not yet been conclusive due to different test parameters and practical approaches used between them for a comparative analysis to be done[18,19]. There are still some unresolved key questions in this this area of research. Most of these centre on understanding how *Wolbachia* interacts with the natural environment and is able to impact viral transmission.

Nevertheless, recently *w*Mel —a *Wolbachia* strain native to *Drosophila melanogaster* was transinfected into *Aedes aegypti* (as it is not a natural host of this bacterium) and has shown promising population replacement ensuring antiviral transmission blocking against the Zika and dengue virus, but this is incomplete[20]. It induces complete uni-directional CI in wild type *Ae. aegypti* and low fitness costs on the host, allowing its penetration and spread in uninfected populations. CI is a pattern of crossing sterility where infected females have a relative reproductive advantage as they can breed with infected and uninfected males, while the uninfected females can only breed successfully with uninfected males[21]. *w*Mel is capable of invading *Aedes aegypti* mosquito populations in the field, although the length of time it may remain prevalent in the population remains unknown. Hence, it is premature to determine the success rate of its viral interference[21-24].

Additionally, *w*AlbB, which is a native *Wolbachia* in *Aedes albopictus,* has a similar viral blocking capacity to *w*Mel in *Ae. aegypti* even though most field studies have focused on the later for release[25]. Interestingly, strains such as the *w*Au—

native in *Drosophila simulans* – cause no CI in their host[26] but have a strong viral inhibition in *Drosophila*[27,28], and are yet to be exploited in future novel transinfections. It is evident that high bacterial density is associated with significant viral suppression in the host[29,30]. Nevertheless, several other ecological factors exist which may alter the density levels of *Wolbachia* in a host for example temperature, other opportunistic agents, lipids and cholesterol levels[31-35].

Vectorial capacity—the ability of a vector to transmit a pathogen to human successfully—is determined by host-vector contact, biting rates, survival age of the vector, extrinsic incubation period (EIP) and vector competence (VC)[36,37]. VC—the ability of a vector to support a given pathogen—is determined by a range of factors including: the vector's genetics, genotype, vector-pathogen interactions and gut-microbiota[37-40]. Furthermore, competition between larvae at their breeding sites, nutritional elements, and temperature could on the other hand play a role in VC. Most importantly the thermal stress subjected to the vector host ensures optimal survival and transmission of the virus from carrier (insect or intermediate host) to receiver (host)[41-43].

Temperature regulates the EIP length and bloodmeal intervals allowing transmission via saliva, which is important in *Ae. aegypti*—a regular feeder (after a few days)[44]. However, the diurnal temperature range could also affect the infection levels and mosquito survival rate[45,46]. This not only applies to the mosquitoes but may also play a role in the fluctuation of the *Wolbachia* densities within them and may therefore also affect the viral-blocking potential[47,48]. Such limitations could inhibit *Wolbachia*-mediated antiviral inhibition in mosquitoes [49,50] and may act as a drawback in the release strategies against dengue and Zika in field studies. The

temperature cycling regimes used in the previous studies were somewhat basic, and may not accurately reflect water temperatures in the field breeding sites.

The aedine mosquito grows in containers and environmental habitats experiencing fluctuating daily temperatures. Recent studies have shown that exposure to heat stress on the developmental stages of the *Aedes aegypti* results in significantly decreased *Wolbachia* density[51,52]. One of the studies by Ulrich and colleagues, showed that there is a noted decrease in *w*Mel density when a temperature regime of 12 hours at 28.5°C followed by 12 hours at 37.5°C is applied in a laboratory setting[52]. Another study by Ross and colleagues indicated a fall in the density of *w*Mel and *w*MelPop (a pathogenic strain of *Wolbachia* also known as popcorn due to its hyper-population inside the host tissues) on exposure to heat patterns between 27°C and 37°C[51]. Several speculative theories have been put forward on the mechanisms of density change such as the deformation of cell membranes which could compromise *Wolbachia* replication[53].

We therefore hypothesized that by subjecting mosquito larvae (a less mobile more localised stage compared to adults) to a temperature pattern based on field collected breeding site temperature data, we could more closely mimic actual field conditions. A comparison between *w*Mel and *w*AlbB could assist in providing a projection of the real-life situation during release of these strains. We show for the first time the effect of heat stress on vectorial capacity. We later challenged three lines of *Ae. aegypti* lines (*w*Mel, *w*AlbB and wild type) with Semiliki Forest Virus (SFV) after two consecutive generations of heat treatment. We found a highly significant 100-fold reduction in the viral blocking ability in *w*Mel under thermal stress, compared to no significant reduction in the relatively resilient *w*AlbB line.

Furthermore, we screened for maternal leakage/ loss after back crossing the wild type males with heat-treated females from the *w*Mel and *w*AlbB lines. We noted an imperfect vertical transmission pattern in the case of heat-treated *w*Mel females after the second generation in comparison to complete (100%) transmission of *w*AlbB. Ovarian tissue tropism via fluorescent microscopy and both tissue dissections (ovaries, midguts and salivary glands) and whole-body density checks using qPCR confirmed a significant decrease in the heat sensitive *w*Mel as compared to thermostable *w*AlbB. Clearly, this study shows that thermal biology is key in selection of robust and stable *Wolbachia* strain suited to high-temperature geographic areas and field-viral-blocking schemes.

Materials and Methods

Cell culture maintenance and counting

Three types of Aag2 cells (Aag2 (control), Aag2-wMelPop and Aag2-wAu) were grown in Schneider's medium with 10% foetal bovine serum (FBS) (Invitrogen) and 10% tryptose phosphate broth (PAN Biotech, Wimborne Dorset, UK) antibiotics: 1000U/ml, Penicillin and 1mg/ml Streptomycin. They were kept in culture by splitting them regularly prior to any experiments and plated in 24 well plates at final concentration of 2.3 X 10^5. The density was confirmed using a haemocytometer and placed in Panasonic Sanyo Incubation chambers (Panasonic Biomedical Sales Europe, Leicestershire, UK) with temperature ranges of 28°C, 32°C and 36°C respectively once they grew up to approximately 70% density/confluency. Images of the various cells under heat treatment were captured using the EVOS® FL Imaging System (Electron Microscopy Sciences, Hatfield, MA, USA).

Cell Viability Assay

The viability of the cell lines was assessed after incubation using the CellTitre-Glo Luminescent Cell Viability Assay (Promega) following manufacturer's instructions. The method detects the number of viable cells present in the culture based on ATP quantities, an indicator of metabolically live cells. Values from this experiment were tabulated as shown in **Table 1.**

Colony rearing and *Wolbachia* transfection

Clean strains of *Aedes aegypti* wild type lines (WT - non-infected with *Wolbachia*) were obtained from a colony retrieved from Selangor State, Malaysia in

1960s. They were maintained at 27°C at relative humidity of 70% with a 12 hours' day versus night cycle[54]. *Aedes aegypti* with *w*Mel and *w*AlbB were obtained from previously infected lines[55,56]. The larval stages were fed on fish pellets (Tetramin, Tetra, Melle, Germany) while adults on a 10% sucrose diet. Blood feeding was done using a Hemotek artificial membrane feeding system (Discovery Workshops, Accrington, UK) and eggs were later collected on damp filter papers (Grade 1 filter paper, Whatman plc, GE healthcare, UK). The eggs were allowed to dry for five to ten days and then hatched in water containing 1g/L bovine liver powder (MP Biomedicals, Santa Ana, California, USA).

Exposure to different temperature patterns

The selected temperatures were adopted from larval breeding sites of *Aedes aegypti* (water storage drums) by Hemme et al[57] for the months of March, May and October in Trinidad (a tropical region) which nearly resembled the daily temperature variations of Selangor State, Malaysia from Weather Underground forecast (www.wunderground.com/weather-forecast/MY/Kuala_Lumpur.html?theprefvalue=0) after overlaying the two graphs on each other (superimposition) (**Fig. 1**). Eggs were hatched from *w*AlbB and *w*Mel strains under simulated larval breeding site temperatures (27°C-control) at 12 hours' day versus night cycle under heat pressure in a Panasonic MLR-352-H Plant Growth Chamber Incubator (Panasonic, Osaka, Japan). This entailed a temperature program using the values shown in **Fig. 1**.

Upon hatching, larvae were collected and reared in white plastic trays (11.5 X 16.5 X 5.5 cm) filled with one litre of (≥ 48 hours) distilled water and larval food whereby a maximum of 200 larvae occupied one tray (this was done in three

replicates per test parameter). Water temperatures were checked using the HOBO® Temp™ Data Logger (HOBO, Bourne, Massachusetts, USA). The larval water was constantly changed after every two days to ensure a bacterial sterile condition. Larval survival was recorded prior to each collection from the heat chamber. Control larvae were reared under normal insectary conditions (constant 27°C, ~70% relative humidity and 12hr: 12hr light: day cycle).

Density determination via qPCR

Genomic DNA was extracted from whole adult bodies and dissected tissues (ovaries, guts and salivary glands) following the method adopted from Veneti et al[58]. In the case of density determination only adults from day 5-post pupal eclosion were used and for dissections 6 pools per tissue type (3 tissues per pool). The DNA was diluted to 100ng/μl using NanoDrop spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). A BioRad 96 real-time PCR detection system was used (BioRad, Hercules, California, USA) with 2 X SYBR- Green mastermix (Biotool, Houston, Texas, USA). Relative quantification was used in the analysis of bacterial density over a standard curve of a vector containing single copies of HTH (a conserved region of *Ae. aegypti* Homothorax) gene and WSP (*Wolbachia* surface protein). Primers specific for quantification of transinfected lines—*w*AlbB and *w*Mel are shown in **Table 2** and were normalised against HTH copies. Prior to setting up the qPCRs, all the primers were checked for efficiency via serial dilutions, which resulted in standard curves with efficiency values greater than 95%. The following thermocyclic conditions were used in this set up: - 95°C – 5mins; 45 cycles of 95°C –

15secs; 60°C – 30secs, followed up with a melt step of 65°C for 5secs and 95°C for 50secs.

Vertical Transmission

The transmission of *Wolbachia* from mother to offspring was assessed in *w*Mel and *w*AlbB infected females, where transinfected lines were crossed to wild-type males in pools of 10 males and 10 females. This ensured genetic variability amongst the lines and minimised an inbreeding depression. A bloodmeal was given to the females and put into single matrilines (individualised) for oviposition (or iso female lines). Eggs from the 10 females were hatched and 5 randomly selected larvae in each case (that is a total of 100 in each line) and their DNA screened via standard PCR using strain specific primers shown in **Table 3**.

Tissue Tropism using Fluorescent *in situ* Hybridisation (FISH)

Ovarian dissection of adults 5-days post emergence was done in Phosphate Buffer Saline (PBS) solution and immediately transferred into a tube containing Carnoy's fixative (chloroform; ethanol; acetic acid, 6:3:1) and fixed overnight at 4°C. Samples were rinsed in PBS and then transferred into 6% hydrogen peroxide in ethanol for 72 hours at 4°C. This was followed by incubation in a hybridization solution containing: 25% 20 X SSC, 0.2% (w/v) Dextran Sulphate, 2.5% Herring Sperm DNA, 1% (w/v) DTT, 1% Denhardt's solution, 50% formamide and 100ng/ml of the universal probe (**Table 4)**. Hybridization of the sample and the probes were left overnight in a dark-damp box at 37°C.

Fluorescent microscopy on whole-body mosquitoes was done using the method adopted from Koga et al[59] with slight modifications. The mosquitoes had

their wings and legs removed and post-fixed in Carnoy's solution overnight at 4°C. Transferring them into 10% hydrogen peroxide in 6% alcohol for 2 weeks quenched autofluorescence. They were then rehydrated in Phosphate Buffer Saline with Tween-20 (PBST) containing: 1XPBS and 0.3% Tween-20 (1–2 hours).

Samples were then rinsed twice in a solution containing: 5% 20 X SSC, 0.015% (w/v) DTT, and then twice in a solution of 2.5% SSC, 0.015% (w/v) DTT in dH2O, with each wash performed at 55°C for 20 minutes. The hybridised sample were then placed on a slide containing a drop of VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, California, USA) and were visualised immediately using a Zeiss LSM 880 confocal microscope (Zeiss, Oberkochen, Germany). Imaging was done in the blue (DAPI) and green (Alexa Flour 488) channels to allow discrete visualization of the *Wolbachia* specific 16S ribosomal RNA probes used to stain for *w*Mel and *w*AlbB and wild-type ovaries. Images were analysed with ImageJ (NIH, USA).

Viral blocking capacity using Semiliki Forest Virus

Semliki Forest virus (SFV) was obtained from Public Health England culture collections and later propagated on C6/36 cells to a final injection concentration of $1.78x10^{13}$ plaque forming units per millilitre (PFU/ml). The choice of the strain was due to its ability to reach high infection loads within the mosquito host after infection. A collection of fifty 5-day old female mosquitoes from each transfected line and wild-type line were orally blood-fed using the membrane feeder on a viremic bloodmeal. They were then immediately transferred into an incubator set at 27°C and at 12-hour day/night cycle for recovery. Any dead mosquitoes after recovery were omitted from

the analysis and an approximate tally in all treatment options was recorded. They were left for ten days prior to RNA extraction and virus quantification by qRT-PCR. RNA was extracted after grinding the samples in TRI Reagent (Sigma-Aldrich, Missouri, USA) using 1mm solid-glass beads (Sigma-Aldrich, Missouri, USA) in a Precellys 24 Lysis and Homogenization Unit (Erlangen, Bavaria, Germany) for 5,500xg for 5secs. Synthesis of cDNA was done using 1μg of total RNA and the All-In-One cDNA Synthesis SuperMix (Biotool, Houston, Texas, USA). A one to ten dilution was carried out on the cDNA prior to a qRT-PCR, which normalised values from primers targeting the non-structural proteins of the virus against the ribosomal protein S17 (RpS17) housekeeping gene (**Table 2**).

Statistical analysis

All data analysis and graphs design was done in Rstudio (version 0.99.489) (Rstudio Inc, Boston, Massachusetts, USA) and GraphPad Prism v.7 (GraphPad Software Inc, La Jolla, California, USA). A two-way analysis of variance (ANOVA) was used to compare the effect of different temperature treatments and *Wolbachia* density and comparisons between treatments and controls had their adjusted P values using multiple comparisons via Tukey's honest significant difference (HSD) and Dunn's multiple comparison test. *Wolbachia* density levels were analysed using the parametric paired *t*-test (if normally distributed) or non-parametric Wilcoxon—Mann—Whitney U test and Kruskal-Wallis test in the case of multiple groups in viral challenge, as they were not normally distributed as outlined by Shapiro-Wilk Test.

Results

Effect of heat on *Wolbachia* density after two successive generations

Studies have focused on using *Wolbachia* as a biological-control tool, although the effects of ecological and environmental factors are an important consideration in the success of this alternative strategy. In this study unlike the highly artificial temperature regimes used in previously published work[51,52], we simulate environmental heat conditions using field-collected temperature data (**Fig. 1**).

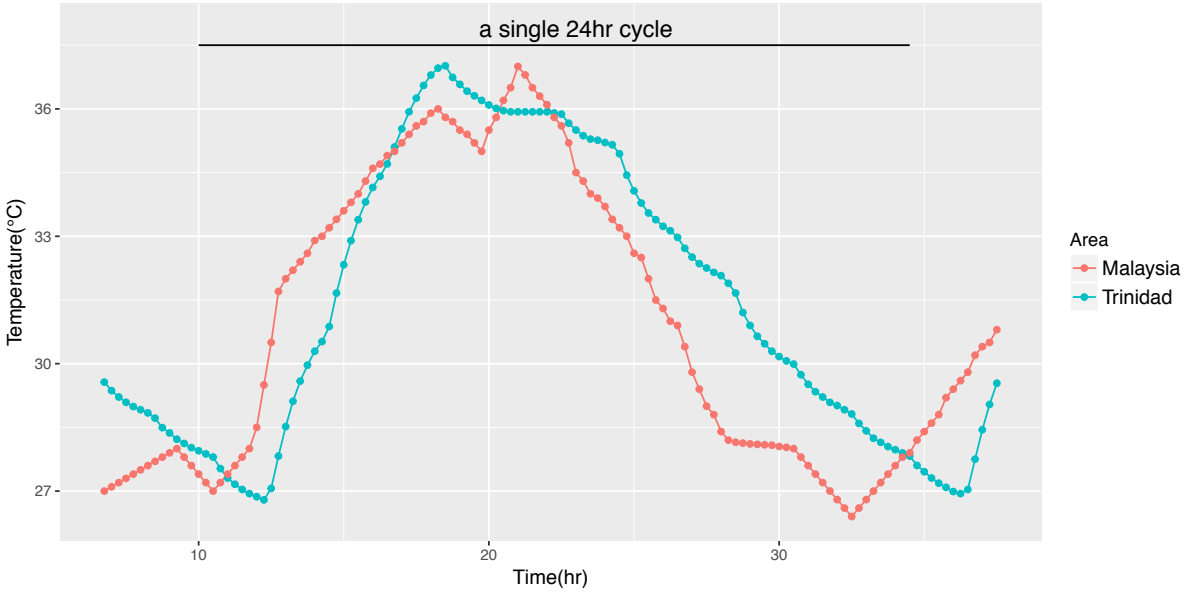
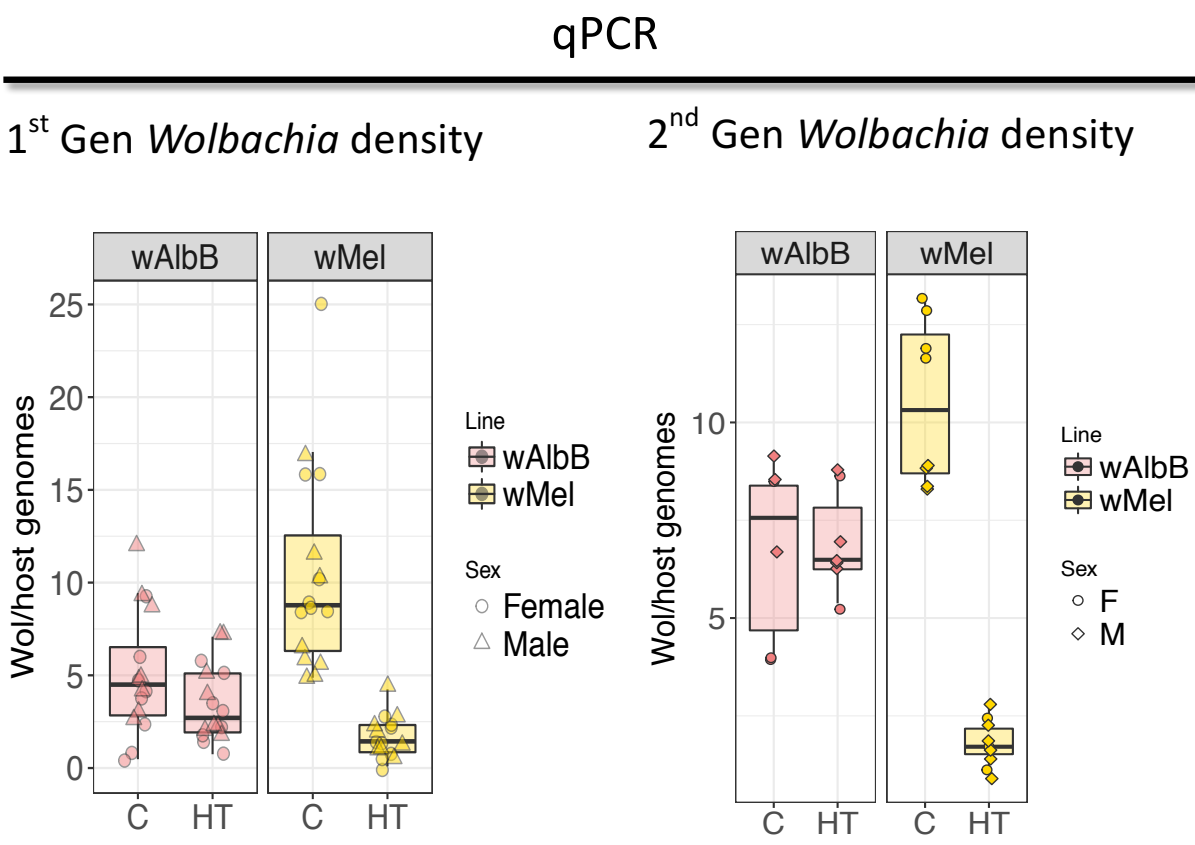


Figure 1: A superimposition of simulated diurnal temperature fluctuations from *Ae. aegypti* larval breeding sites. This indicates temperature recorded from surface probes places on both plastic and steel containers from Trinidad simulated in an incubator and read by a HOBO® Temp™ Data Logger (shown in blue) combined for the months of March to May 2005 and October 2007 and 2008. March had a temperature range of 23.4°C to 29.1°C, while May had 26.0°C to 36.0°C. Whereas there were more variations in temperature in the month of October as compared to March and May as they recorded a range of 22.4°C to 32.8°C. The underlying graph is the daily temperature fluctuations of Malaysia for April 19, 2016 from Weather Underground (shown in red), which superimpose on those in Trinidad.

Larvae from the *w*Mel, *w*AlbB and wild-type lines were set-up in two simultaneous heat-treatment conditions; either exposed to heat stress or under controlled laboratory temperature (28°C) (**Fig. 2**). There was a significant drop in *Wolbachia* density in the *w*Mel line following heat-treatment compared to the control (P= 0.0001554, Mann-Whitney *U* test) dropping from an average of ten *Wolbachia* copies per host genome to two copies (post-heat stress). No significant change was noted in heat-treated *w*AlbB compared to controls (P= 0.57737, Mann-Whitney *U* test). These results corroborate previous studies, which used temperature regimes on *w*Mel and *w*AlbB and noted that *w*AlbB was heat stable[51,52].



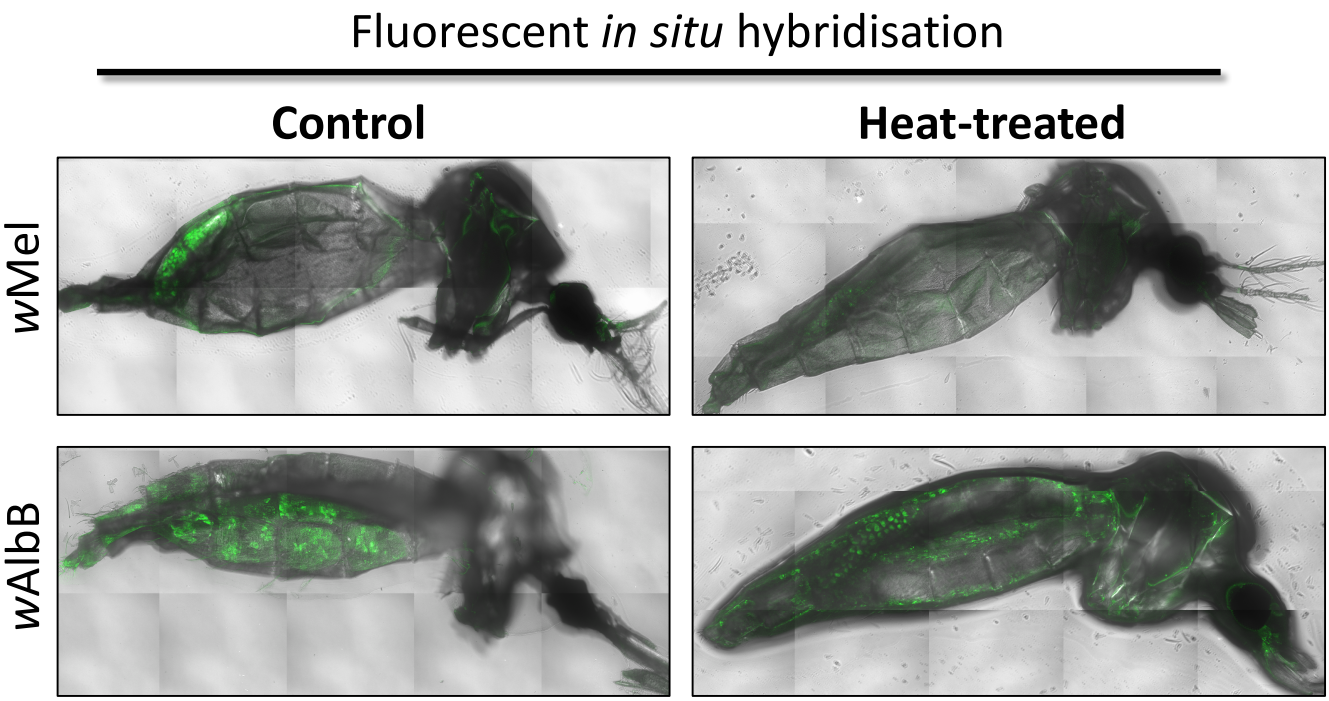
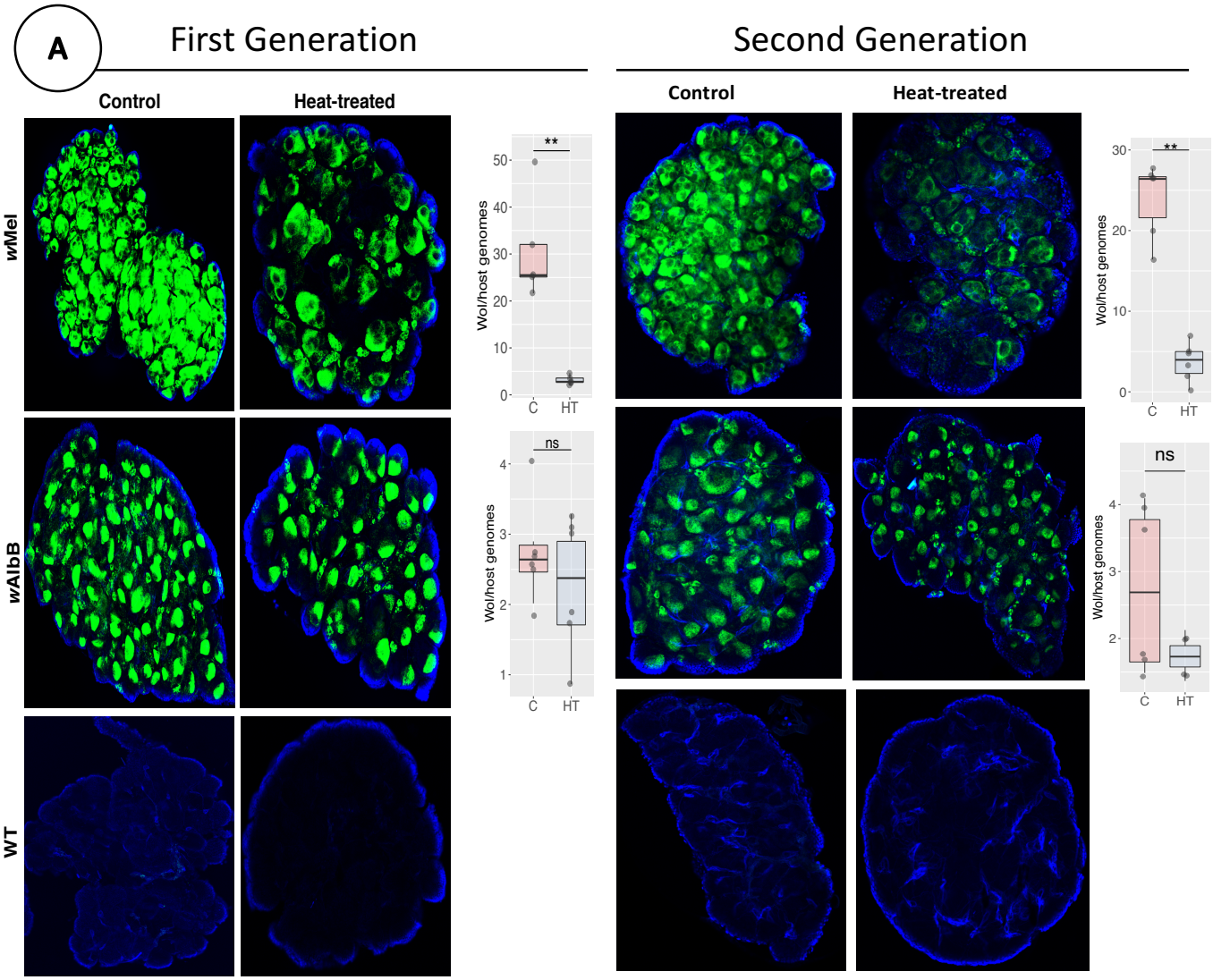


Figure 2: *Wolbachia* density dynamics across lines due to heat stress in 1st and 2nd generation of infected *Aedes aegypti*. a) qPCR data for whole-body density determination from *w*AlbB and *w*Mel larvae were subjected to heat stress using a simulated temperature patterns. 5-day-old adult females were later screened for density variations via qPCR, data shown by the respective boxplots. The C stands for the control lines while the HT stands for the Heat-treated lines. The central mark on the boxplot represent the median point while the scattered shapes represent individual data points. In the *w*AlbB line, accumulative data points of both males and females between the control and heat-treated had P>0.1(ns), whereas in the *w*Mel line the P<0.0001(***). b) Whole-body fluorescent microscopy on emerging females collected at the same time point as the qPCR indicating density of *Wolbachia* (signal seen in green) across the body tissues, with most fluorescent staining brightly around the ovaries as seen in *w*Mel control and *w*AlbB heat-treated panels.

In addition to whole-body qPCR quantification, was performed qPCR analysis on dissected ovarian, midguts and salivary gland tissues. *Wolbachia* density was determined from the aforesaid tissues after two generations of larval heat treatment in a fraction of females not subjected to viral challenge (**Fig 3**).



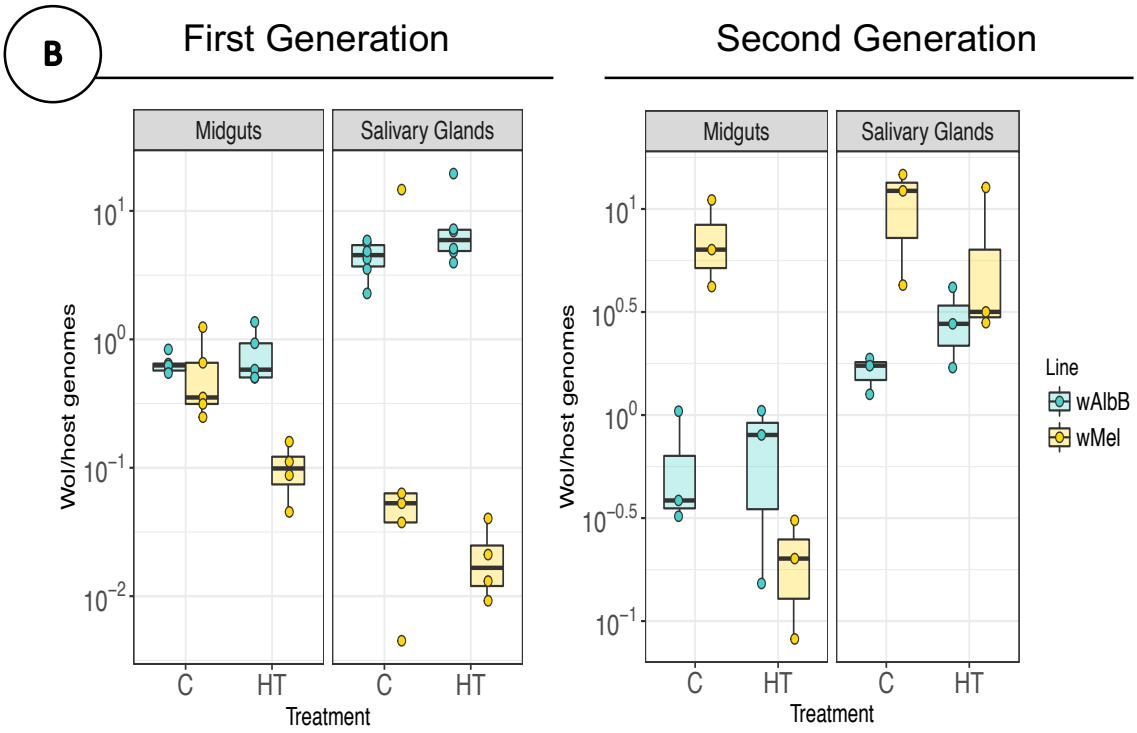


Figure 3: Fluorescence microscopy versus qPCR density quantification of 5-day-old female dissected tissues. a) The first panel on the left shows images of ovaries from controls and first-generation heat-treated lines and adjacent *Wolbachia* qPCR quantification after normalising the host gene— HTH (*Aedes aegypti*) against WSP (*Wolbachia* surface protein) gene represented by boxplots with a median point. The control panel indicates a cluster of ovaries with the green fluorescence representing the *Wolbachia* specific probes and the blue signal from the periphery is the DAPI stain (nuclear staining). There is a change in the signal intensity between the *w*Mel control and heat-treated as compared to the *w*AlbB line. This correlates with density results shown on the immediate right of each panel. A Wilcox-Mann-Whitney *U* test on the density values gave P values as annotated on the boxplots, ** means P > 0.001 whereas ns means P > 0.1 (not significant). The second panel shows results for the second-generation round of experiments. b) First and second generation 5-day-old females were dissected and in addition to the previously quantified ovaries, midguts and salivary glands were also separately screened for density fluctuations using qPCR as they as key tissues in viral transmission. Using Dunn's multiple comparison test, P values within lines and between treatment

options in the lines (C –Control, HT –Heat-treated) was greater than 0.5 (ns). *Wolbachia* density within individual lines, after heat treatment in certain tissues gave a P > 0.05(ns) but a comparison of density in the salivary glands of *w*AlbB HT in relation to *w*Mel HT gave P > 0.0001(***)

Comparison of *Wolbachia* density in the salivary glands after thermal application shows a significant difference between the *w*AlbB and *w*Mel (P = 0.0009, Dunn's test). Ovarian dissections and whole-body fluorescent microscopy showed a correlation in the density drop as shown in **Fig. 2 & 3**. There was a significant drop in the quantity of *Wolbachia* in heat-treated *w*Mel ovaries compared to the control (P= 0.0058, t test) whereas *w*AlbB showed no significant difference (P= 0.1183, t test).

Maternal leakage occurs in heat sensitive *w*Mel strain after heat treatment

In the third-generation offspring (generated after mating second-generation infected females from *w*AlbB and *w*Mel infected lines with wild type males) a 20% drop was observed (using a standard PCR) in maternal transmission rate in *w*Mel after heat treatment compared to controls (**Fig. 4**). In the case of *w*AlbB, there was no change in the percentage of vertically transmitted *Wolbachia* between the heat-treated and controls.

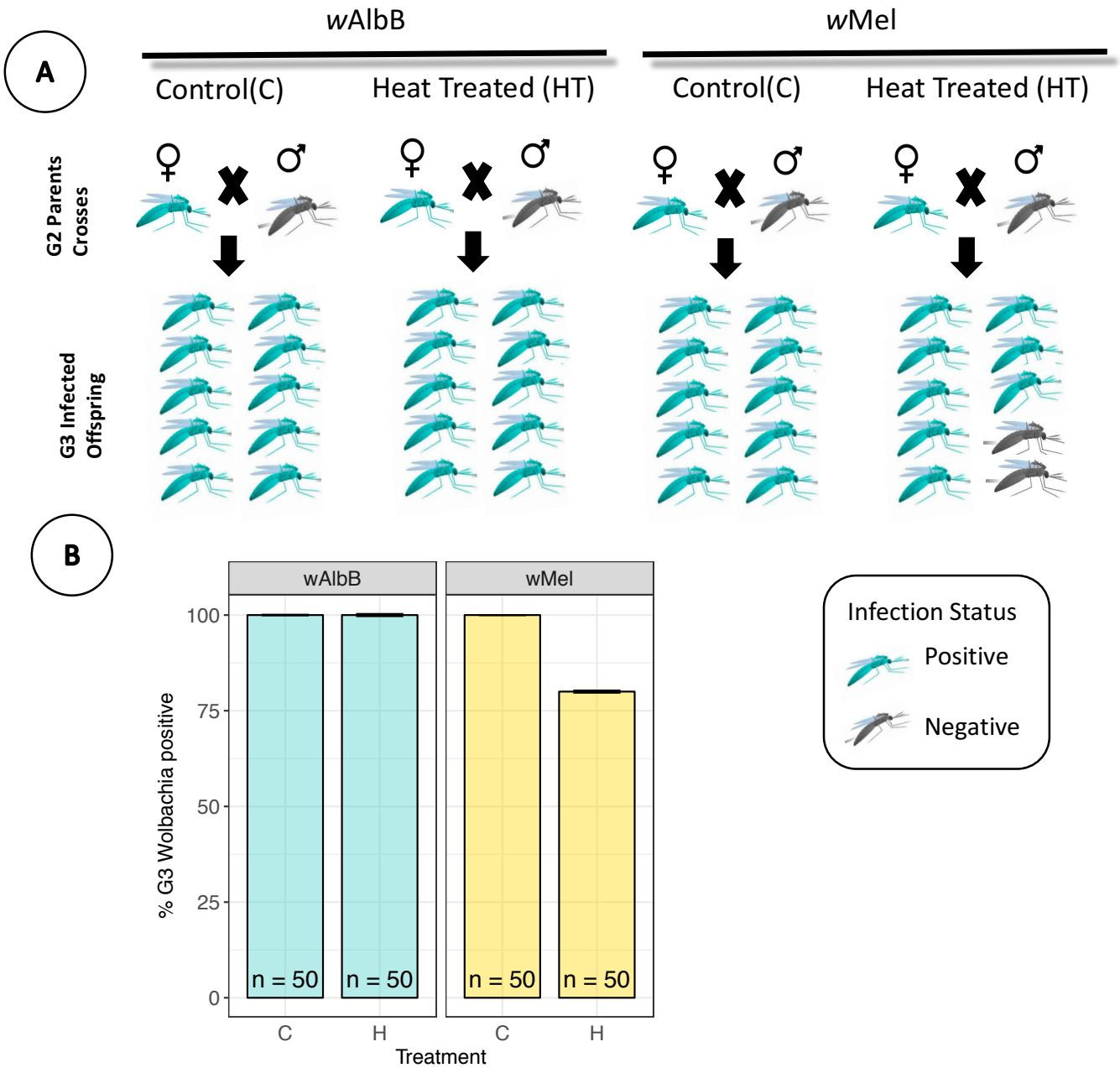


Figure 4: The effect of heat treatment on maternal transmission. a) After the second generation of heat treatment, females from the respective groups we crossed with wild type *Ae. aegypti* male (free of any *Wolbachia* strain). The figure shows the outcome in the offspring after backcrossing the lines. The legend key with mosquito of two colours: - the light blue signifies the positive / *Wolbachia* infected mosquito and the black represents the negative / wild type or *Wolbachia* clean line is shown. b) Standard PCR on the offspring from the respective single female lines was plotted in bar graph as a percentage count of the total of positives per individual

female whereby 10 females (5 larvae from each female) were screened for the presence or absence of *Wolbachia* in the progeny. Error bars were generated as shown in Table 5.

Furthermore, quantification of *Wolbachia* in the females (the ones crossed with the wild type males to give the third-generation offspring) two weeks later after leaving them under the control temperature (27°C) showed a recovery in density. There was no significant difference in the *Wolbachia* density between the control and heat-treated both in the *w*Mel and *w*AlbB strain (*w*Mel—P =0.8929, *w*AlbB—P =0.8310, Tukey's HSD test) (**Fig. 5**).

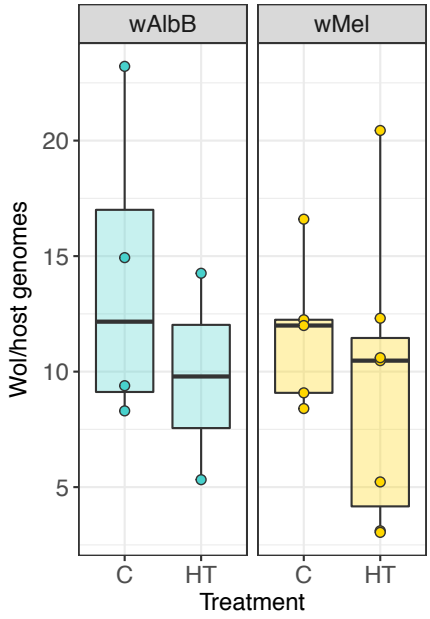


Figure 5: Recovery and stabilisation of *Wolbachia* density after heat treatment. The females from the second-generation maternal transmission experiment that survived two weeks later after laying eggs were reared under the control temperature (27°C) and screened using qPCR for density dynamics. The boxplots show dots that signify an individual surviving female (which were few (n<10)) across both lines and a median point in each case. Wilcox-Mann-Whitney *U* Test across the various groups of heat treatment gave a P > 0.5 (ns).

Thermal stress lowers viral blocking capacity of *w*Mel as compared to *w*AlbB

Recent studies showed the consequence of heat stress on *Wolbachia* density but no study so far has measured the resulting effect on vectorial capacity[51,52]. Here we give a report for the first time of the effect of temperature on *Wolbachia* virus-blocking capacity after two generations of heat treatment using Semiliki Forest Virus (SFV). SFV is a single-stranded positive-sense alphavirus (type II) that is easily manageable in a laboratory setting and provides a convenient model for RNA flaviviruses such as Zika and dengue as well as the alphavirus chikungunya to which it is related.

SFV quantification was performed via qPCR to check the copy number of SFV against a host gene (RpS17) ten-day post-infection. Female mosquitoes showed significant blocking in *w*Mel and *w*AlbB control lines as compared to wild type (wt) control ($P < 0.0001$, Dunn's test) (**Fig. 6 a**). Comparison of the SFV infection rate within individual lines (that is *w*Mel, *w*AlbB and wt) across the heat-treated and control group showed no significant difference in SFV loads despite the thermal intervention ($P > 0.9999$, Dunn's test). Nevertheless, the percentage count using a bargraph denoting the presence and absence of SFV infected mosquitoes shows that in case of heat treatment, *w*Mel heat-treated mosquito lost their blocking ability from as low as 0.001(negative) to nearly 1 copy of SFV per mosquito cell—a 100-fold reduction (**Fig 6 b**). This essentially means that in a whole-body there is an increased chance of the mosquito being infective (virus present in the salivary glands).

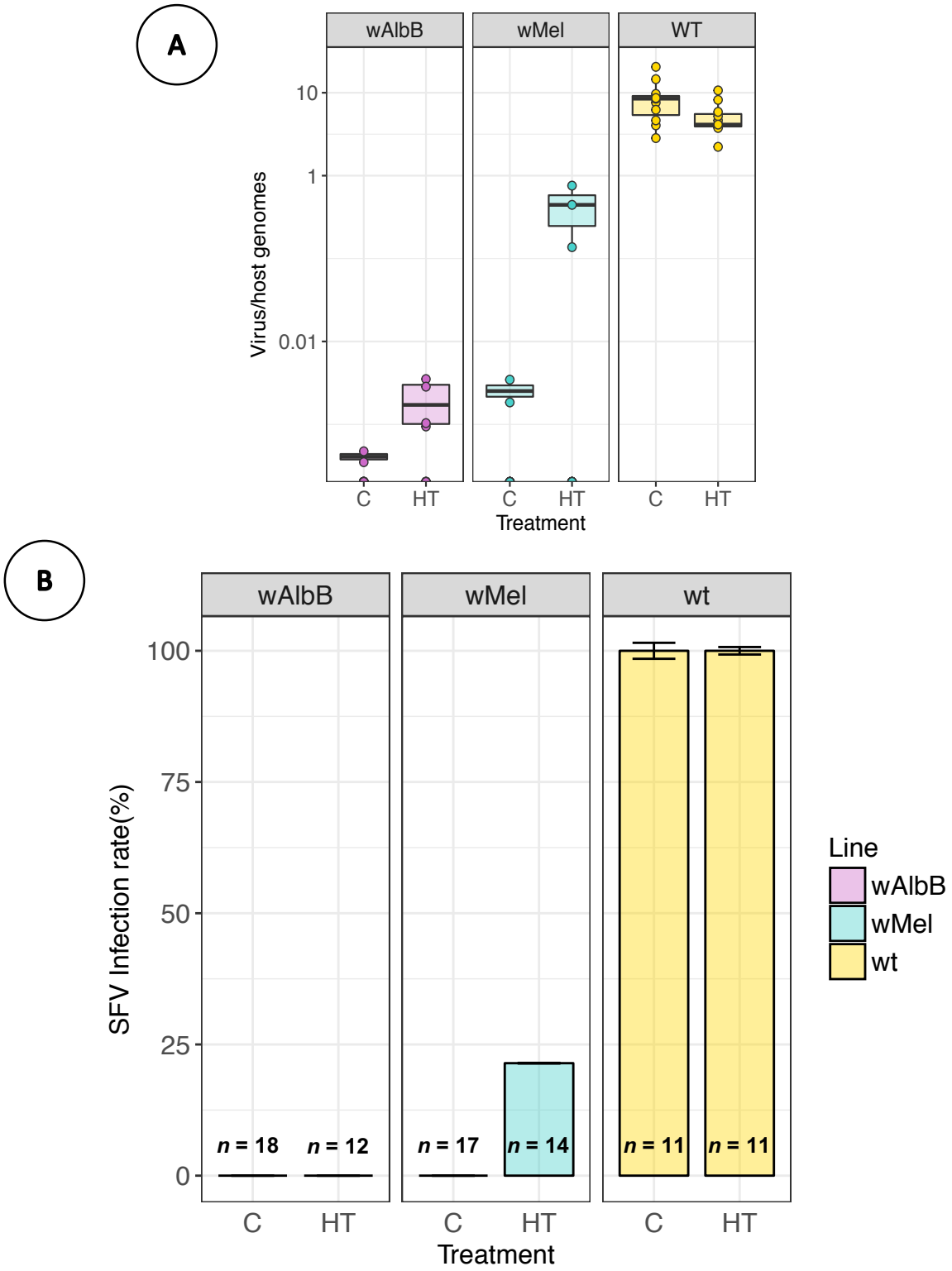


Figure 6: ***Wolbachia*** quantifications in midguts and salivary glands and SFV titres on ***Wolbachia Ae. aegypti*** lines after heat stress. a) SFV quantification from second-generation 5-day-old females challenged with SFV and then RNA extracted 10 days post infection for titre determination via qPCR. The boxplot indicates the titre retrieved from individual females under various line the *w*AlbB, *w*Mel and WT (wild type) *Ae. aegypti*. Presence of very low readings as 0(complete blocking) together with Dunn's comparison test – P value greater than 0.5 (ns), in each line across heat

treatment, except in the case of wAlbB and wMel infected lines in relation to WT with P > 0.0001(****) led to the development of a bargraph b) with percentage count of infected versus uninfected where SFV values below 0.01 were considered "negative" and those above 0.01 were "positive" in each treatment group for clear correlation; the error bars used were derived from standard deviation values from single female SFV titre values from figure b) per respective group. *n* represents the number of females used in the final viral quantification.

Wolbachia **infected** ***Aedes*** **tissue culture assays: Cell viability and density**

In an additional set of experiments, we used *Aedes aegypti* derived mosquito cell cultures lines (Aag2 cell lines) to examine whether heat treatment induced similar effects on *Wolbachia* density *in vivo*[60]. *w*MelPop and *w*Au infected cell lines were subjected to heat regimes (28°C -control temperature, 32°C, 36°C) (**Fig. 7**). There were no significant differences in density between the three temperature ranges (P > 0.999, Tukey's HSD test).

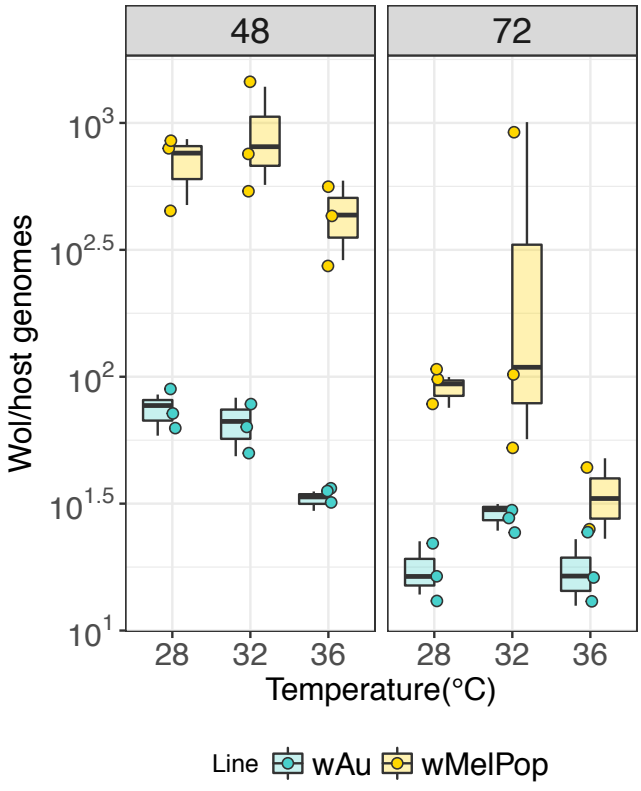


Figure 7: ***Wolbachia*** density in heat-treated Aag2 ***w***MelPop, Aag2 ***w***Au. The different cell lines were subjected to both 48 and 72hrs of different temperature ranges from the control temperature (28°C) to higher heat brackets of (32°C and 36°C). qPCR density quantification values were plotted as shown in the boxplots with median points for both *w*MelPop and *w*Au. Tukey's HSD test on 48hr time and 72hr interval, across both cell lines generated a $P > 0.9$(ns).

However, from the boxplots it appears as though there is a gradual decrease, but this is not significant statistically. This change in *Wolbachia* slightly resembles the adult mosquito colony lines, however, the experiment being done in sharp temperature changes may have caused the slight change in expected results. Therefore, using simulated temperatures instead of temperature regimes and similar strains Aag2 derived cell lines (*w*Mel and *w*AlbB) may give a clearer picture of the *Wolbachia* density alterations.

Cell viability data showed no significant difference in ATP levels (a signature for active metabolism in cells) across the various heat treatments compared to control conditions at 28°C in the Aag2 control cells (all values had $P > 0.05$, t test) (**Fig. 8**). This indicates that heat fluctuations did not lead to cell line mortality. However, this may be partly a consequence of no significant changes in density of *w*Au and *w*MelPop Aag2 cell lines across all temperatures ($P > 0.999$, Tukey's HSD test). Light microscopy on the mosquito cell lines suggested that the cells were healthy despite the change in temperature after 48 hours. In the case of 72 hours, the cell lines looked unhealthy, and an accumulation of cell debris (at 36°C) were seen—an indication of the fitness cost the two high density strains (*w*MelPop and *w*Au) can have[60] (Appendices **Fig. 9**)

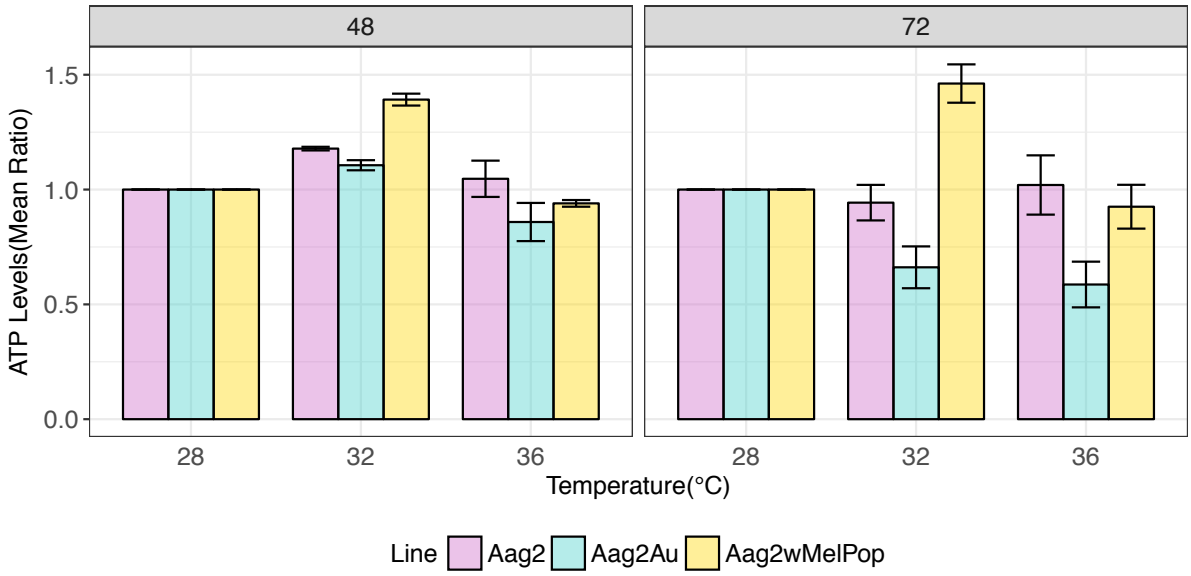


Figure 8: Viability assay for Aag2, Aag2wMelPop and Aag2wAu across 28°C, 32°C and 36°C for 48 and 72hrs. The first box shows bar graphs of the three cell lines at different temperatures for the first 48hrs of heat treatment exposure. The second box contains bar graphs of the different cell lines after 72hrs of heat treatment in a separate experiment run concurrently with the 48hrs. Error bars are generated from standard deviations from the mean ratio values (shown in **Table 4**). Using a two-tailed, pairwise T-test to compare ATP levels between the control temperature (28°C) and the elevated temperatures (32°C, 36°C), the P value was greater than 0.05(ns).

Discussion

Temperature affects many aspects of insect biology (physiology) including interactions with other microbes[44] and this can be a complex factor in host-pathogen interactions. Moreover, temperature can undergo highly significant fluctuations in the natural environment, which are often not reflected in constant temperatures used in normal laboratory settings[61]. This ecological surrounding ultimately affects the insect host resulting in strong manifestation of physiological changes, in this case the reduction in *Wolbachia* density[62,63]. Given that strong viral blocking is linked to high density *Wolbachia* strains; a drop in bacterial density equates to a failure in the success of a given strain as an effective viral control agent in the natural environment[29,30].In general, it is important to consider both abiotic and biotic factors when investigating the host-pathogen interactions for a full understanding of their relationship. Results from experiments under laboratory conditions can prove misleading when the experimental system is considered under the more complex field conditions[64,65].

Successful vertical transmission of *Wolbachia* from one generation of mosquito population to the next allows its high frequency and stability in the natural environment. Its persistence increases the chances of an effective and promising blocking strategy. Results from previous publications suggested far higher rates of maternal leakage (up to 100%), here we show that there is a more modest loss in maternal transmission in the *w*Mel as compared to the resistant *w*AlbB, for the more realistic temperature regime used. The difference in results from our study and previous papers highlights the need to mimic diurnal temperature ranges in the respective release sites rather than focusing on temperature brackets with sharp

changes, which may be over exaggerated as reported in previous studies[51,52].

Recovery of *Wolbachia* is an aspect of bacteria's biology that remains unknown. The mechanism with which different strains or *Wolbachia* as a whole maintains itself within the host is quite understudied. This may explain the slight occurrence of maternal leakage that was noted in the *w*Mel line under heat treatment, however, further investigation is needed to understand how recovery occurs under various strains of *Wolbachia* and mosquito hosts.

Previous studies have quantified viral infectivity and transmission in mosquitoes based on saliva[66] or either head containing salivary glands (viral infectivity) and carcass (viral dissemination) in determining effects of temperature on viral blocking against the dengue virus[67]. Here we use the whole bodies, which in its own is a combined component of both transmission and infection capacity of the vector but in totality represent the vector capacity of the mosquito and this may be a limitation in the overall detection of SFV titres. Despite this, results from this study are important as the virus infects and penetrates the midgut barrier into the hemolymph and finally the salivary gland; where it is passed on to another host during the next bloodmeal. The more the human hosts present at the vectors predisposition, the more the viral agent persists in the population, especially in the case of *Ae. aegypti* which is a regular feeder (after 1-2 days)[36]. For that reason, future studies should strive to quantify virus from tissues of interest (midguts and salivary glands) as this increases the chances of viral detection in their respective tissues of viral interest as opposed to whole body quantification.

Visually, there seems to be a significant difference in SFV infection rate between the heat-treated and control group in the *w*Mel line but lacks statistical

power due to scarcity of data points (see **Fig 6a**). Evidently, setting up a perfect viral challenge experiment is quite technical. There are many underlying limitations such as time constraint as mosquitoes are fed and later monitored over a time-course of ten days post-infection (to achieve a complete EIP cycle), the high levels of mosquito mortality witnessed after a viremic bloodmeal which lower the overall number collected in the end for a strong statistical correlation. Lastly, this low count in turn affects the number of qPCR non-zero data points (see **Fig 6a**), which can be only counteracted by starting off with relatively large numbers to ultimately achieve a reasonable number of surviving individuals.

Finally, investigating the effect of simulated field temperatures on other factors affecting vectorial capacity such as EIP and on other RNA viruses such as Zika, dengue and chikungunya could be informative in broadening the scope of viral blocking. Hence, future studies should adopt more sensitive approaches coupled with a thermal component in order to increase understanding of these interactions.

Conclusion

This study clearly shows the importance of accurately simulated field temperatures on determining how environmental conditions will affect *Wolbachia*-mediated viral protection. It puts a clear boundary between diurnal temperature changes and the previously used two-temperature-24-hour regimes in which rapid change between temperatures may itself have caused the stress. We limited these temperature regimes to larvae but it would be highly important to carry out the same set of experiments on adults in a green-house/ semifield setting or an adult adapted incubation chamber.

Moreover, we show that there is a possibility of *Wolbachia* density recovery at the adult stage after heat treatment, which may explain the reduced rate of maternal leakage as opposed to the one recorded in harsh temperature regimes from previous studies. Therefore, it would be interesting to know whether subjecting the adult mosquitoes to higher temperatures, e.g. 40°C (an attainable temperature in Malaysia) in further experiments will reduce the probability of *Wolbachia* recovery or loss.

Thirdly, immune activation occurs due to introduction of *Wolbachia* strains in mosquitoes offering both antiviral and antiparasitic protection[22,25,68-70]. An increased reactive oxidative stress (ROS) alongside the Toll pathway stimulation was seen in *Wolbachia* transinfected *Ae. aegypti* which mediated antiviral blocking[70]. This means that blocking is not solely dependent on *Wolbachia* or host but their mutual interaction; otherwise the two key factors are – *Wolbachia* density and immune stimulation. Hence, understanding host immune mechanism such as autophagy, ROS, viral trafficking mechanisms and heat shock proteins[71] may increase

understanding of the complexity of interaction and allow the development of a concrete theory of how viral blocking is occurring.

In addition, the tripartite interaction between the host, virus and the bacteria has been studied over time, however the evolution dynamics remain unknown[72]. Whether the virus may evolve to overcome antiviral blocking by *Wolbachia* has been argued and the existence of *Wolbachia* in natural field mosquitoes at low density may favour reduced antiviral effects[24]. This has been the case in *Wolbachia* transinfected lines, which are unstable and are unable to achieve stability and/ or high densities within their new hosts. This clearly means that the *w*Mel strain currently under release in field trials may suffers from density instabilities due to heat stress resulting in a viral bottleneck allowing the viral dissemination in endemic areas once more.

In conclusion, this study serves a baseline guide on the selection criteria upon which a more superior and stable strain is chosen for field release. Further studies are still needed to answer the complex interaction that exists between different *Wolbachia* strains and their viral blocking ability as recently it was show that the *Wolbachia* strain is the main determinant of viral interference[73]. Notwithstanding all these theories, the ecological aspects remain crucial in determining which immune activation occurs and how the host, bacteria and virus respond.

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Supporting information (Appendices)

Table 1: Cell Viability Assay: ATP levels across Temperatures (28°C, 32°C and 36°C) in Aag2, Aag2*w*MelPop and Aag2*w*Au Cell Lines

	48hrs									72hrs										T-test value	
	28°C	**32°C**	**Ratio [a]**	**Average [b] ratio**	**Stdev. [c] (ratio)**	**36°C**	**Ratio [a]**	**Average [b] ratio**	**Stdev. [c] (ratio)**	**28°C**	**32°C**	**Ratio [a]**	**Average [b] ratio**	**Stdev. [c] (ratio)**	**36°C**	**Ratio [a]**	**Average [b] ratio**	**Stdev. [c] (ratio)**	**Time**	**28°C vs 32°C**	**28°C vs 36°C**
Aag2	818157	783780	1.17	1.18	0.01	682261	1.02	1.05	0.08	1378580	1555170	1.03	0.94	0.08	1740860	1.15	1.02	0.13	**48hr**	0.26	0.76
	595346	787993	1.18			659581	0.99			1358200	1326500	0.88			1350450	0.89			**72hrs**	0.66	0.88
	594558	794308	1.19			760395	1.14			1801500	1397670	0.92			1537220	1.02					
Average	**669353.7**									**1512760**											
Aag2wMelPop	531414	740209	1.39	1.39	0.03	505563	0.95	0.94	0.01	1586750	1989040	1.38	1.46	0.08	1256920	0.87	0.93	0.10	**48hr**	0.01	0.09
	552873	724303	1.36			500674	0.94			1502620	2103500	1.46			1492930	1.04			**72hrs**	0.06	0.44
	508135	751664	1.42			490383	0.92			1236130	2229800	1.55			1252360	0.87					
Average	**530807.3**									**1441833.3**											
Aag2Au	417538	707118	1.10	1.11	0.02	598368	0.93	0.86	0.08	1832870	1776490	0.76	0.66	0.09	1242370	0.53	0.59	0.10	**48hr**	0.60	0.58
	753195	703855	1.09			569379	0.88			3137810	1356030	0.58			1640020	0.70			**72hrs**	0.26	0.07
	765057	729910	1.13			494439	0.77			2043810	1506530	0.64			1231730	0.53					
Average	**645263.3**									**2338163.3**											

Key

[a] This ratio is obtained by division of values in 32°C or 36°C with the average ATP value from the 28°C of each time point either 48hr or 72hr per respective group (Aag2, Aag2*w*Au and Aag2*w*MelPop).

[b] This is the average of the ratios generated in each category depending on the various calculations carried out as per instructions part [a].

[c] This is the standard deviation retrieved from the deviation in values from the various ratios per individual groups, used in plotting bar graphs in **Fig. 2**.

Table 2: Relative qPCR quantification primers

No.	Name	Sequence	Reference
1	qWSP-F	ATCTTTTATAGCTGGTGGTGGT	[22]
2	qWSP-R	AAAGTCCCTCAACATCAACCC	[22]
3	qHTH-F 43	TGGTCCTATATTGGCGAGCTA	[74]
4	qHTH-R 43	TCGTTTTTGCAAGAAGGTCA	[74]
5	RpS17 F	TCCGTGGTATCTCCATCAAGC	[75]
6	RpS17 R	CACTTCCGGCACGTAGTTGTC	[75]
7	SFV-F	CGCATCACCTTCTTTTGTG	[76]
8	SFV-R	CCAGACCACCCGAGATTT	[76]

Table 3: Primer sequence for standard PCR

No.	Name	Sequence	Reference
1	DNAGyrase*w*AlbBF1	TGAGTTGTTGGTTACAGCTTCC	This study
2	DNAGyrase*w*AlbBR1	TAAAAGTCGCAATTCAGATGGC	This study
3	WSP 81 F	TGGTCCAATAAGTGATGAAGAAAC	[74]
4	WSP *w*Mel/Pop R	CAGCCTGTCCGGTTGAATT	This study

Table 4: Fluorescent probes used in FISH

No.	Name	Sequence	Reference
1	W2	CTTCTGTGAGTACCGTCATTATC	[22]
2	W3	AACCGACCCTATCCCTTCGAATA	[22]

Table 5: Maternal transmission scores after heat treatment

Line	Family No	Control	Standard deviation	Standard error	Line	Family No	Heat-treated	Standard deviation	Standard error
*w*Mel	1	5	0	0*	*w*Mel	1	3	0.9487	0.3*
*w*Mel	2	5			*w*Mel	2	4		
*w*Mel	3	5			*w*Mel	3	5		
*w*Mel	4	5			*w*Mel	4	4		
*w*Mel	5	5			*w*Mel	5	4		
*w*Mel	7	5			*w*Mel	6	3		
*w*Mel	8	5			*w*Mel	7	4		
*w*Mel	9	5			*w*Mel	8	3		
*w*Mel	10	5			*w*Mel	9	2		
						10	5		
*w*AlbB	1	5	0.3162	0.1*	*w*AlbB	1	3	0.7868	0.2973*
*w*AlbB	2	5			*w*AlbB	2	4		
*w*AlbB	3	5			*w*AlbB	3	5		
*w*AlbB	4	4			*w*AlbB	4	5		
*w*AlbB	5	5			*w*AlbB	5	5		
*w*AlbB	6	5			*w*AlbB	6	4		
*w*AlbB	7	5			*w*AlbB	7	5		
*w*AlbB	8	5			*w*AlbB	8			
*w*AlbB	9	5			*w*AlbB	9			
*w*AlbB	10	5			*w*AlbB	10			

* The standard error bars were used in **Fig 8b.**

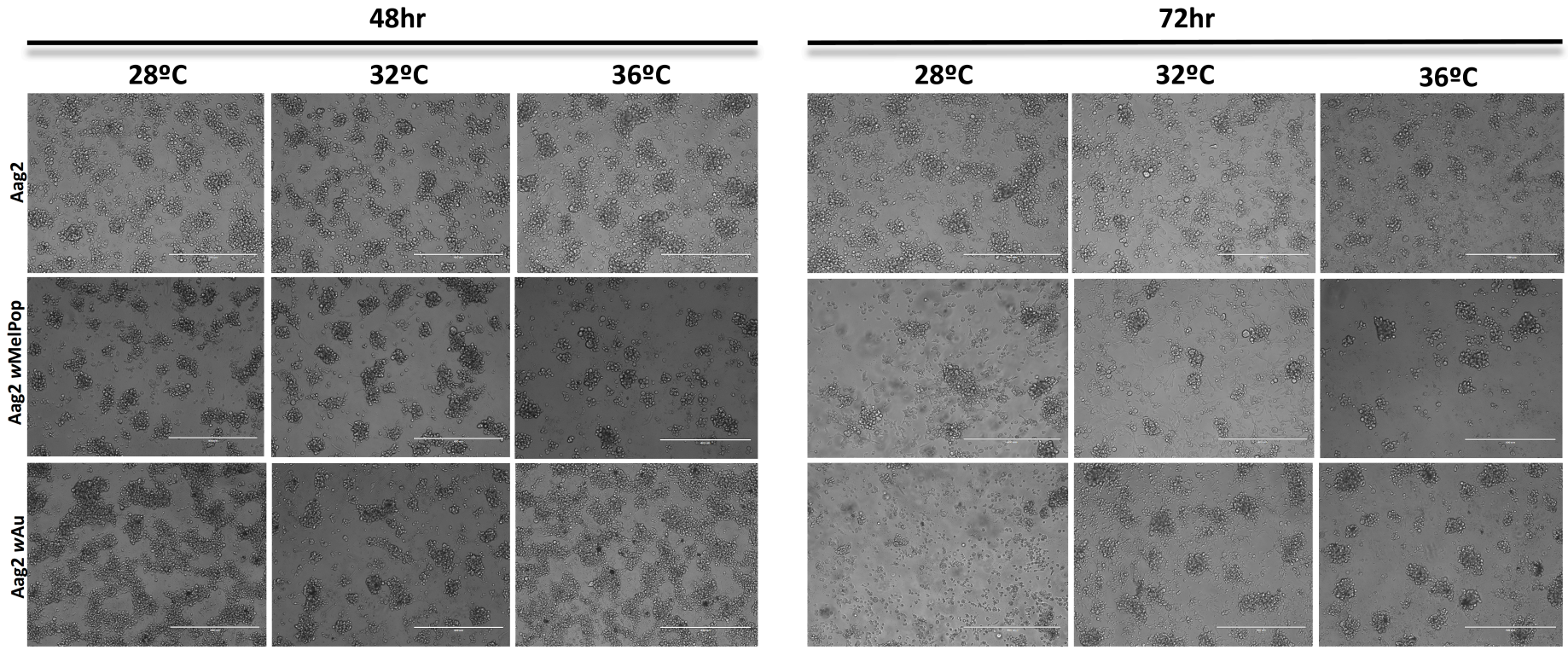

Figure 9: Light Microscopy on Aag2, Aag2 *w*MelPop and Aag2 *w*Au Cell line after 48 and 72hr of heat treatment. on Aag2, Aag2 *w*MelPop and Aag2 *w*Au Cell line after 48 and 72hr of heat treatment. Each panel of 3 cells shows the state of the cells after the time lapse assigned, with heterogeneous forms of clustered *Aedes aegypti* cell lines in the healthy Aag2 control cells. In the case of less healthy cell lines as in the case of 72hrs of heat treatment, there is presence of few clustered cell lines with scanty cell debris (seen in Aag2 *w*MelPop 36°C, 72hr). Images taken at 10X magnification.