Sensitivity of wMel and wAlbB Wolbachia infections in Aedes aegypti Puducherry (Indian) strains to heat stress during larval development

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Abstract

Background: ICMR-Vector Control Research Centre, Puducherry, India, developed two colonies of Aedes aegypti infected with wMel and wAlbB Wolbachia strains called Ae. aegypti (Pud) lines for dengue control. The sensitivity of wMel and wAlbB strains in Ae. aegypti (Pud) lines to heat stress was studied.

Methods: wMel and wAlbB infected and uninfected Ae. aegypti larvae (first to fourth instars) were reared in the laboratory to adults at 26 °C, 30 °C, 36 °C and 40 °C constant temperatures and also 26–30 °C, 26–36 °C and 26–40 °C diurnal cyclic temperatures. The adults were tested for Wolbachia infection. Experiments were also carried out rearing the larvae under simulated field conditions in summer (April and June) under sunlight using fully open and half open bowls and also under sunlight and natural shade.

Results: At 36 °C and 40 °C constant temperatures, complete larval mortality was observed. At 30 °C and 26 °C, no larval mortality occurred, but Wolbachia density was relatively low in wMel infected males compared to control (maintained at 26±1 °C). At diurnal cyclic temperature of 26–40 °C, Wolbachia density was reduced in males of both the (Pud) lines, but not in females. At 26–36 °C, reduction in Wolbachia density was observed in wMel males but not in wAlbB males. At 26–30 °C, no significant reduction in Wolbachia density was observed with wMel and wAlbB strains. In simulated field conditions (April), under sunlight, the daytime water temperature reached a maximum of 35.7 °C in both full and half open bowls. No larval mortality occurred. Wolbachia frequency and density was reduced in wMel-infected Ae. aegypti (Pud) males from both type of bowls and in females from full open bowls, and in wAlbB males from half open bowls. In June, rearing of larvae under sunlight, the first-instar larvae experienced a maximum daytime water temperature of > 38 °C that caused complete mortality. No larval mortality was observed in bowls kept under shade (< 32 °C).

Conclusions: Exposure of larvae to higher rearing temperatures in the laboratory and simulated-field conditions reduced the densities of wMel and wAlbB strains particularly in males, but the impact was more pronounced for wMel strain. The actual effect of heat stress on the stability of these two Wolbachia strains needs to be tested under natural field conditions.

Keywords: Aedes aegypti, Wolbachia, Heat stress, Temperature tolerance, Dengue, India
Background

Dengue, a mosquito-borne, acute febrile illness, is a major public health problem in the tropics and the subtropics worldwide. According to the World Health Organization (WHO), over 129 countries are now endemic to dengue. It is estimated that 390 million dengue infections and 96 million dengue cases occur worldwide annually [1]. In India, outbreaks of dengue have been reported in 28 States and 6 Union Territories. A total of 687,890 dengue cases and 1110 deaths due to dengue infection were reported in the country during 2015–2020 [2]. Since there are no effective vaccines for community immunization and no drugs for treatment, control of the disease vector is the only option available for dengue control [3, 4]. *Aedes aegypti* is the major vector of dengue virus in India, and *Ae. albopictus* is successfully established among wild mosquito populations [15]. Successful invasion of *Wolbachia* into the native *Ae. aegypti* populations at the field sites of Australia, Brazil, Indonesia, Malaysia and Vietnam has been associated with varying levels of reduction in disease prevalence in the treated community. Recently, a cluster randomised trial in Yogyakarta city, Indonesia, demonstrated 77% reduction of virologically confirmed dengue cases post-release of *Wolbachia* mosquitoes [16]. Non-randomised controlled field trials in Indonesia [17] and Australia [18, 19] showed respectively 76% and 96% reduction of dengue incidence. In city-wide field trials, *Wolbachia* deployments caused 69% reduction of dengue cases in Brazil [20] and about 86% in Vinh Luong city, Vietnam [21].

To explore the alternate method of control of dengue transmitted by *Ae. aegypti*, Indian Council of Medical Research-Vector Control Research Centre (ICMR-VCRC), Puducherry, India, in collaboration with World Mosquito Program (WMP), Monash University, Australia, has successfully developed two new Indian *Wolbachia*-infected *Ae. aegypti* Puducherry (Pud) release lines through backcross experiments. The newly developed Indian *Ae. aegypti* (Pud) release lines infected with wMel or wAlbB *Wolbachia* strains are to be tested in field at a pilot scale to select a suitable strain for Indian conditions. Prior to field release, it is essential to assess the fitness of the release lines in the laboratory, as these mosquitoes should survive under field conditions for successful establishment of *Wolbachia* among the wild mosquito population. Besides, there are various environmental factors that would affect successful establishment of the inherited *Wolbachia* infections among the wild mosquito populations. Sensitivity to temperature is one such factor that could potentially limit the invasive capacity of a *Wolbachia* transinfected mosquito strain and also its ability to inhibit virus replication, thereby transmission.

Recent studies showed that *Wolbachia* strains in *Ae. aegypti* were vulnerable to higher temperatures [22–25]. Immature stages of *Ae. aegypti* grow in container habitats such as flower pots, water tanks, earthen pots, plastic barrels/drum, gutters, automobile tires, discarded utensils/containers, bottles, cans, scrap, etc., available in domestic and peri-domestic environments [26–28]. However, *Ae. aegypti* gravid females prefer to lay their eggs in shaded containers, the immature stages are also commonly found in containers that are fully exposed to sunlight [29, 30]. Ulrich et al. [22] reported that larval development of *Ae. aegypti* at higher water temperatures can experience attenuation in the *Wolbachia* levels. Exposure of larvae to high rearing temperature has been reported to reduce the ability of *Wolbachia* to induce cytoplasmic incompatibility and also the density of *Wolbachia* in adults [22, 25]. Therefore, in the current study, the ability of wMel and wAlbB *Wolbachia* strains in *Ae. aegypti* (Pud) lines to tolerate higher temperatures was studied under laboratory and simulated-field conditions.
Methods

Mosquito strains and colony maintenance
Eggs of wMel and wAlbB-infected Ae. aegypti Australian (Aus) strains were imported from WMP, Monash University, Australia, and reared at ICMR-VCRC, Puducherry, India. By backcrossing the females of wMel or wAlbB-infected Ae. aegypti (Aus) strains with wild (field caught) Ae. aegypti Puducherry (Pud) males over six generations, two new release lines, viz., wMel Ae. aegypti (Pud) and wAlbB Ae. aegypti (Pud), were developed and maintained for over 20 generations and at every generation, males of the release lines were outcrossed with 10% wild caught males. Eggs of uninfected wild Ae. aegypti (Pud) strain were collected using ovitraps from different sites of Puducherry, reared to adults, fed with human blood and allowed to oviposit. The F1 eggs were used for temperature sensitivity studies.

Temperature sensitivity studies under laboratory conditions
The tolerance of wMel and wAlbB infections to two temperature regimens was studied under laboratory conditions. In the first regimen, first-instar larvae of wMel/wAlbB Ae. aegypti (Pud) release lines and uninfected wild Ae. aegypti (Pud) line were exposed to temperature maintained constantly at 30 °C, 36 °C and 40 °C up to pupal stage. In the second regimen, the larvae were reared at diurnal cyclic temperatures of 26–30 °C, 26–36 °C and 26–40 °C to pupae.

The eggs of the wMel/wAlbB Ae. aegypti (Pud) release lines and the uninfected wild Ae. aegypti (Pud) line were hatched in cooled boiled (deoxygenated) water containing brewer’s yeast (0.2 g/l). Batches of 25 first-instar larvae of each line were released separately into 500-ml glass beakers containing 300 ml of tap water and the beakers were placed inside a water bath-stirred (14 l capacity, temperature range: 5–90 °C; EQUITRON Medica Pvt Ltd Mumbai, India) till the larvae pupated. The water baths were set to maintain temperature constantly at 30 °C or 36 °C or 40 °C or at daytime cycling temperatures of 26–30 °C, 26–36 °C and 26–40 °C. Four replicates (each with 25 larvae) were kept for each temperature regime and for each line. Simultaneously, larvae of wMel/wAlbB Ae. aegypti (Pud) and uninfected wild Ae. aegypti (Pud) were maintained constantly at 26 °C±1 °C outside a water bath as controls for each experiment. Larvae were fed with fish food, Tetramin tropical tablet @ 2.00 mg per larva, during the experiments. Water temperature inside the water bath and the glass beakers was recorded using submerging data loggers (Tiny tag aquatic, Gemini data loggers, UK). Five-day-old emerged adults from all the experiments were screened for Wolbachia frequency and density. The experiments were replicated twice using different batches of first-instar larvae.

Temperature sensitivity studies under natural sunlight
Temperature sensitivity studies were also carried out under sunlight during summer months (April and June) at ICMR-VCRC premise, Pondicherry district, Union Territory of Puducherry. Pondicherry has a tropical climate with moderate variation of temperature and rainfall. Summer starts in April and lasts up to early June when maximum temperature may reach 41 °C (106 °F). The average maximum temperature ranged from 28 °C in January to 37 °C in May and the average minimum temperature fluctuated between 20 °C (January) and 27 °C (May). The average annual rainfall is about 1260 mm and almost 68% of it falls during October to December.

Experiment I—exposure to sunlight with full open/half open bowls
In this experiment, we used two types of plastic bowls (500 ml capacity; 14 cm diameter and 6.5 cm depth), fully open and half open (partially covered). The bowls were partially covered using chart sheet paper. Batches of 50 first-instar larvae of each line were separately released into plastic bowls (fully/half open) containing 300 ml of tap water and placed under sunlight. Three replicates were maintained for each line and type of bowls. The bowls were covered with nylon net at sunset (18.00 h) to prevent the wild mosquitoes from ovipositing and the net covers were removed the next day morning.

Experiment II—exposure to direct sunlight and natural shade
In this experiment, batches of 50 first-instar larvae of wMel/wAlbB Ae. aegypti (Pud) release lines and wild Ae. aegypti (Pud) line were released separately into plastic bowls (500 ml capacity) containing 300 ml of water and placed under sunlight. Three replicates (each with 50 larvae) were maintained for each line. Simultaneously, batches of 50 first-instar larvae of each line (in three replicates) were released separately into 500-ml plastic bowls with 300 ml of water and placed under tree shade. For both experiment I and II, larvae of wMel/wAlbB Ae. aegypti (Pud) and uninfected wild Ae. aegypti (Pud) were maintained at a constant temperature of 26 °C±1 °C as control. Water temperature in the bowls was recorded at hourly intervals using submerging data loggers for the entire duration of the experiment.
In both experiments I and II, equal quantities (2.00 mg/ larva) of larval food (crushed Tetramin tablets) were used to feed the larvae until their pupation. On day 5 and 6, pupae from each replicate were collected and returned to the insectary and placed inside labelled Bugdorm cages (15 × 15 × 15 cm) for emergence. The emerged adults were provided with 10% sucrose solution (soaked in cotton wool) and maintained at a constant temperature of 27±2 °C and a relative humidity of 80±10% up to day 5 post-emergence. Five-day-old, non-blood-fed adults (both males and females) of each line and temperature regimen were screened for Wolbachia frequency and density.

Screening for Wolbachia frequency and density
The frequency and the density of Wolbachia infections in Ae. aegypti lines were estimated using real-time PCR. Individual mosquitoes were screened for the presence of Wolbachia by multiplex real-time Taqman PCR assay, using the primers and the probes targeting WSP gene for wMel, Ankyrin repeat domain gene for wAlbB, respectively. Simultaneously, RPS gene (ribosomal protein), specific for Ae. aegypti, was used as positive control. Density of Wolbachia in individual mosquitoes was estimated using Comparative C_{T} (2−ΔΔCT) method following the standard operating procedure (SOP) of WMP, Monash University, Australia, September 2018, on “Screening of Wolbachia (wMel and wAlbB) in adult mosquitoes using triplex qPCR (96 well)” [31].

Statistical analysis
Data were expressed as mean (SD) and range (minimum, maximum). Mann-Whitney U test was used to determine the difference in Wolbachia density between the experimental and control groups at different temperature regimens. Paired t-test was used to compare the temperatures between the full and half open bowls. P-value < 0.05 was considered statistically significant. All statistical analyses were done in statistical software STATA 14.2 version (College Station, TX, USA).

Results
Tolerance to constant temperatures in laboratory
On exposure to the constant temperature of 40 °C, complete mortality of larvae of both wMel and wAlbB Ae. aegypti (Pud) release lines and also of the wild Ae. aegypti (Pud) was observed. Similarly, when exposed to 36 °C maintained constantly, all the larvae of wAlbB and wild (Pud) lines, except seven larvae of wMel, died and on screening the adult mosquitoes emerged from those seven alive larvae (4 ♀ and 3 ♂); none were found positive for Wolbachia. On exposure to the temperature constantly maintained at 30 °C and also to controls (maintained at a constant temperature of 26 °C±1 °C), Wolbachia frequency was 100% in both males and females of wMel and wAlbB Ae. aegypti (Pud) release lines.

The Wolbachia density (Table 1) in both males and females of wMel and wAlbB Ae. aegypti (Pud) release lines exposed to the constant temperatures of 30 °C did not differ significantly from the corresponding controls (maintained at 26±1 °C) (wMel female: U=25, Z=0.74, P=0.46; wAlbB male: U=30, Z=0.21, P=0.83; wAlbB female: U=15, Z=1.79, P=0.07 by Mann-Whitney U test), except in wMel males (Pud), in which the density was significantly lower than the control (U=5, Z=2.84, P=0.005) (Table 2).

Tolerance to diurnal cyclic temperatures in laboratory
The frequency of Wolbachia was 84.6–100% in both males and females of wMel and wAlbB Ae. aegypti (Pud) release lines at the diurnal cyclic temperatures of 26–40 °C, 100% (except one replicate of wMel female that showed a frequency of 92.3%) at 26–36 °C and also 100% (except one replicate of wMel (Pud) females which had a frequency of 91.7%) at 26–30 °C.

At the diurnal cyclic temperature of 26–40 °C, the Wolbachia density in wMel (Pud) and wAlbB (Pud) males was significantly lower than the controls (wMel male: U=7, Z=2.63, P=0.009, wAlbB male: U=12, Z=2.1, P=0.036), whereas in the females, the density was not

Table 1 Wolbachia frequency and density in wMel and wAlbB Ae. aegypti (Pud) release lines on exposure of their larvae (first instar) to constant temperature of 30°C compared to 26±1°C (Control)

| Strain     | Temp (constant) | Replicate | No. of larvae exposed | No. emerged/ screened | Wolbachia frequency (%) | Average Wolbachia density (range) |
|------------|----------------|-----------|-----------------------|-----------------------|-------------------------|----------------------------------|
| wMel (Pud) | 30 °C          | 8         | 200                   | 54 46                 | 1.56±0.39 (1.08–2.18)   | 10.16±3.91 (5.59–17.05)          |
|            | 26±1°C Control | 8         | 200                   | 53 47                 | 2.73±0.77 (1.57–3.81)   | 11.61±3.76 (7.54–17.75)          |
| wAlbB (Pud)| 30 °C          | 8         | 200                   | 51 49                 | 28.95±5.28 (22.43–39.86)| 22.99±6.78 (15.68–34.64)         |
|            | 26±1°C Control | 8         | 200                   | 50 50                 | 30.00±7.51 (20.69–38.51)| 29.52±6.90 (16.39–39.46)         |
significantly different from the controls (wMel female: U = 29, Z = 0.32, P = 0.75; wAlbB female: U = 20, Z = 1.26, P = 0.21) (Fig. 1, Table 2). At 26–36 °C, in wAlbB (Pud) males (wAlbB male: U = 21, Z = 1.16, P = 0.25) and in females of both the release lines, the density did not differ significantly from the controls (wMel female: U = 19, Z = 1.37, P = 0.17; wAlbB female: U = 16, Z = 1.68, P = 0.09). However, there was a significant reduction of the density in wMel (Pud) males compared to the control (wMel male: U = 11, Z = 2.2, P = 0.03) (Fig. 1, Table 2). At 26–30 °C, the density of wMel in both males and females did not differ significantly from the control (wMel male: U = 29, Z = 0.32, P = 0.75; wMel female: U = 25, Z = 0.74, P = 0.46). While the density in wAlbB males was not significantly different from the control (U = 23, Z = 0.95, P = 0.34), the difference in the density between wAlbB (Pud) females and the corresponding control was at the statistical limit (wAlbB female: U = 13, Z = 2.00, P = 0.05) (Fig. 1, Table 2).

Temperature tolerance on exposure to sunlight
Temperature tolerance was studied by exposing the larvae directly to direct sunlight. In the first regimen, two types of bowls, full and half open (partially covered), were deployed. The mean minimum water temperature was 26.7 ± 0.66 °C (range: 26.02–27.7 °C) in the full open bowls and 26.8 ± 0.67 °C (range: 26.12–27.8 °C) in the half open bowls kept under sunlight. The mean

### Table 2 Wolbachia density in wMel and wAlbB Ae. aegypti (Pud) release lines on exposure in larval stage to temperatures maintained at constantly and different ranges of diurnal cyclic temperatures in laboratory and under sunlight (natural) compared to the respective line exposed to a constant temperature of 26 ± 1 °C (control) in laboratory.

| Temperature/condition | Experiment | Control (26 ± 1°C constant) |
|------------------------|------------|-----------------------------|
|                        | Replicate  | n  | Mean (SD) | Min.–max. | Replicate  | n  | Mean (SD) | Min.–max. |
| **wMel male**          |            |    |           |           |            |    |           |           |
| 26 °C to 40 °C*        | 8          | 200 | 1.33 (0.92) | 0.39–2.41 | 8          | 200 | 9.17 (7.67) | 2.05–19.14 |
| 26 °C to 36 °C*        | 8          | 200 | 2.20 (0.94) | 1.14–3.91 | 8          | 200 | 3.07 (0.56) | 2.42–3.93  |
| 26 °C to 30 °C         | 8          | 200 | 3.99 (0.89) | 2.44–4.85 | 8          | 200 | 3.85 (1.82) | 2.07–6.44  |
| 30 °C                 | 8          | 200 | 1.56 (0.39) | 1.08–2.18 | 8          | 200 | 2.73 (0.77) | 1.57–3.81  |
| Full open bowls*      | 3          | 150 | 2.05 (0.54) | 1.66–2.66 | 4          | 100 | 16.02 (3.47) | 11.38–19.14 |
| Half open bowls*      | 3          | 150 | 0.75 (0.24) | 0.47–0.93 | 4          | 100 | 16.02 (3.47) | 11.38–19.14 |
| **wMel female**        |            |    |           |           |            |    |           |           |
| 26 °C to 40 °C        | 8          | 200 | 7.36 (4.48) | 1.50–12.17 | 8          | 200 | 8.29 (3.67) | 4.57–13.75 |
| 26 °C to 36 °C        | 8          | 200 | 6.20 (3.90) | 2.55–11.76 | 8          | 200 | 7.29 (4.28) | 3.19–12.44 |
| 26 °C to 30 °C        | 8          | 200 | 11.44 (5.40) | 1.35–16.70 | 8          | 200 | 10.67 (4.08) | 4.63–15.90 |
| 30 °C                | 8          | 200 | 10.16 (3.91) | 5.59–17.05 | 8          | 200 | 11.61 (3.76) | 7.54–17.75 |
| Full open bowls*      | 3          | 150 | 10.91 (0.93) | 9.89–11.72 | 4          | 100 | 5.05 (0.55) | 4.57–5.80  |
| Half open bowls       | 3          | 150 | 5.57 (4.16) | 3.05–10.37 | 4          | 100 | 5.05 (0.55) | 4.57–5.80  |
| **wAlbB male**        |            |    |           |           |            |    |           |           |
| 26 °C to 40 °C        | 8          | 200 | 31.68 (6.03) | 24.29–38.68 | 8          | 200 | 37.35 (4.21) | 28.47–41.15 |
| 26 °C to 36 °C        | 8          | 200 | 29.67 (7.27) | 17.39–36.23 | 8          | 200 | 23.96 (11.09) | 13.65–40.43 |
| 26 °C to 30 °C        | 8          | 200 | 18.10 (14.61) | 3.61–33.44 | 8          | 200 | 29.63 (2.88) | 25.53–33.80 |
| 30 °C                | 8          | 200 | 28.95 (5.28) | 22.43–39.86 | 8          | 200 | 30.00 (7.51) | 20.69–38.51 |
| Full open bowls       | 3          | 150 | 32.99 (4.81) | 28.23–37.85 | 4          | 100 | 35.82 (5.70) | 28.47–40.39 |
| Half open bowls*      | 3          | 150 | 23.46 (0.91) | 22.77–24.50 | 4          | 100 | 35.82 (5.70) | 28.47–40.39 |
| **wAlbB female**      |            |    |           |           |            |    |           |           |
| 26 °C to 40 °C        | 8          | 200 | 24.09 (11.63) | 12.23–39.69 | 8          | 200 | 31.06 (8.11) | 20.87–43.07 |
| 26 °C to 36 °C        | 8          | 200 | 24.25 (10.01) | 10.90–43.56 | 8          | 200 | 17.77 (11.14) | 9.07–41.56  |
| 26 °C to 30 °C        | 8          | 200 | 19.35 (4.46) | 14.77–27.91 | 8          | 200 | 23.61 (2.93) | 20.03–28.65 |
| 30 °C                | 8          | 200 | 22.99 (6.78) | 15.68–34.64 | 8          | 200 | 29.52 (6.90) | 16.39–39.46 |
| Full open bowls       | 3          | 150 | 34.53 (5.15) | 29.33–39.63 | 4          | 100 | 26.22 (5.19) | 20.87–32.67 |
| Half open bowls       | 3          | 150 | 23.65 (4.80) | 20.25–29.15 | 4          | 100 | 26.22 (5.19) | 20.87–32.67 |

* Statistically significant

† Number of larvae exposed
maximum water temperature was 38.54 ± 2.24 °C (range: 35.7–41.2 °C) and 38.1 ± 2.04 °C (range: 35.6–40.4 °C) in full and half open bowls, respectively. Overall and over time, the diurnal fluctuations of water temperature did not differ significantly between full and half open bowls (t(5) = 0.422; P = 0.673, by paired samples t-test).

After the exposure of larvae to sunlight in full open bowls, the frequency of wMel ranged from 68.2–85.0% in adult males and 82.4–91.6% in females. It was 100% in both males and females of wAlbB (Pud) Ae. aegypti. When larvae were reared in half open (partially covered) bowls under sunlight, the Wolbachia frequency ranged from 20.83 to 80.95% in wMel males and 28.57–66.66% in females. It was 100% in wAlbB (Pud) males and females.

The Wolbachia density in the two release lines and controls after exposure to sunlight in full and half open bowls is presented in Fig. 2 and Table 2. When compared to the control (maintained constantly at 26 °C ± 1 °C), there was a significant reduction of Wolbachia density in wMel males in both types of bowls (full open: U = 0, Z = 2.12, P = 0.03; half open: U = 0, Z = 2.12, P = 0.03) and also in wAlbB males exposed in half open bowls (U = 0, Z = 2.12, P = 0.03) (Table 2). However, no significant reduction was observed in wAlbB female in both types of bowls (full open: U = 1, Z = 1.17, P = 0.08; half open: U = 4, Z = 0.71, P = 0.480 and also in wAlbB males in full open bowls (U = 3, Z = 1.06, P = 0.29).

**Temperature tolerance in sunlight vs shade**

In this experiment, larvae were exposed to sunlight without any shade on the bowls and to full natural shade in June, the warmest month of the year. On day 1, the ambient temperature at 06.00 h was 27 °C and it reached a maximum of 41.8 °C at 12.00 h. From 12.00 to 14.00 h, the temperature was > 40 °C. The water temperature in the experimental bowls kept under sunlight was in the range of 38.2 to 39.3 °C at 12.00 h, and on day 1, complete mortality of first instar larvae was observed in these bowls. In the bowls kept under shade, the water temperature reached a maximum of 30.6 °C during the daytime and no larval mortality was observed. The experiment was discontinued because of complete mortality of first instar larvae in bowls kept under sunlight and the larvae kept under shade were also not reared to adults to screen the Wolbachia frequency and density.

**Discussion**

In this study, we examined the sensitivity/tolerance of wMel and wAlbB infections in Ae. aegypti (Pud) lines to heat stress under laboratory and simulated field conditions. Rearing of larvae (first to fourth instars) of
Wolbachia-infected and -uninfected *Ae. aegypti* (Pud) at the constant temperatures of 36 °C and 40 °C resulted in complete/near complete mortality. At 30 °C constant temperature, there was no larval mortality, but reduction of Wolbachia density was observed in *wMel* *Ae. aegypti* adult males. However, exposure of Wolbachia-infected and -uninfected *Ae. aegypti* larvae to constant rearing temperatures may not simulate/represent the actual field conditions; these experiments provided a critical thermal maximum (≥36 °C) beyond which mortality of *Ae. aegypti* larvae occurs.

At the diurnal cyclic temperature of 26–40 °C, Wolbachia density was reduced in males of both the release lines, but not in females, indicating that Wolbachia infection in males was sensitive to heat stress. Furthermore, the reduction of density was observed only in *wMel* males but not in *wAlbB* males at 26–36 °C, which points out relatively more sensitivity of *wMel* to heat stress. *Wolbachia* strains in *Ae. aegypti* have been reported to differ in their response to heat stress [23, 32]. Rearing of *wMel*- and *wMel*-Pop-CLA-infected *Ae. aegypti* (Aus strain) larvae at diurnal cyclic temperature of 26–37 °C reduced the density of *Wolbachia* in adults drastically; in contrast, *wAlbB* infection was maintained at high density [23]. Exposure of larvae to rearing temperature fluctuated between 27 °C and 37 °C reduced the density of *wAlbA*, *wAlbB* and *wMel*; however, the impact was more pronounced for *wMel* [32]. These findings were from the laboratory studies and it was not clear whether the effects of heat stress on *Wolbachia* are transient and will be restored back in the absence of heat stress. Foo et al. reported that *Wolbachia* density got partially recovered in female offspring of parents that experienced heat stress under laboratory conditions [24].

Experiments under simulated field conditions were carried out during summer (April and June). In the first experiment, two types of bowls, full and half open (partially covered), were used to rear larvae under natural sunlight. Half open bowls were deployed to provide partial shade to the larvae while rearing, expecting that the temperature of rearing water should be less compared to full open bowls. However, no significant difference in the daily fluctuations of rearing temperature was observed between the two types of bowls probably because of small size of the containers (500 ml capacity, with 300 ml of water) used for the experiment. Though the chart paper used to partially close the bowls provided shade, it might have also limited the dissipation of heat from the bowl water. During the experiment with full/half open bowls conducted in April, first-instar larvae were exposed to a maximum water temperature of 35.7 °C and second, third, and fourth instars and pupae to a maximum

![Fig. 2](image-url)
daytime water temperatures of 37.5 °C, 37.9 °C, 40.4 °C and 41.2 °C, respectively. No larval and pupal mortalities were observed. However, there was a reduction of Wolbachia frequency and density in wMel-infected Ae. aegypti (Pud) males and females. For wAlbB infected Ae. aegypti, there was a reduction of density in wAlbB males and not in females, indicating wAlbB infection in females was less sensitive to heat stress. In experiment I (exposure in full/half open bowls), the maximum daytime water temperature in the bowls on day 1 was 35.7 °C, which did not kill any first-instar larvae. However, in experiment II (exposure to sunlight/shade) conducted in June, first-instar larvae experienced a maximum daytime water temperature that fluctuated between 38.2 °C and 39.3 °C in different replicates, which was on the higher side. This caused complete (100%) mortality indicating the critical thermal point and that first-instar larvae were most vulnerable to heat stress.

In the current study, reduction of Wolbachia density was observed at a high rearing temperature under laboratory as well as simulated field conditions and the results were consistent with the earlier observations [22, 23, 25, 32]. The thermal death point for Wolbachia-infected and -uninfected Ae. aegypti larvae was ≥36 °C under both laboratory and simulated field conditions. Comparison of densities of wAlbB and wMel in Ae. aegypti (Pud) release lines showed wMel was more sensitive to higher temperatures, while wAlbB was more resilient. Similarly, comparison of Wolbachia density between male and female mosquitoes indicated that infection in males was highly sensitive to diurnal cyclic temperatures, matching the observation by Ross et al. [23]. It has been reported that wAlbB strain has a better thermostability profile compared to wMel in mosquito larvae and the strain has been selected for deployment in Kuala Lumpur, Malaysia, for dengue control [15]. However, the temperatures set in the laboratory experiments were meant to mimic larval habitat temperatures in the field, but did not truly represent those experienced by mosquitoes in field conditions [18].

For a successful field release strategy, Wolbachia infections should persist at high frequencies and block virus transmission under field conditions for many years following deployment [33]. Recent studies reported that Wolbachia strains are vulnerable to high temperatures [22, 23, 25, 32]. Aedes aegypti larvae are commonly found in container habitats in the peri-domestic environment, often experiencing wide diurnal fluctuations of temperature, especially in habitats that are exposed to sunlight. The effectiveness of the strategy could therefore be influenced by environmental temperature, which may decrease Wolbachia frequency and density, thereby reducing the ability of Wolbachia to invade and persist in the population and block virus replication. Despite being sensitive to heat stress, wMel strain has been released successfully in several tropical countries where high temperatures may have a deleterious effect on Wolbachia. In large-scale city-wide field releases, spatial and seasonal heterogeneity in wMel invasion was observed. In a quasi-experimental trial in Niterói, Brazil, deployments of wMel-infected Ae. aegypti mosquitoes during 2017–2019 resulted in heterogeneous invasion and spread of wMel in to the local Ae. aegypti populations at an infection frequency of 33–90% by March 2020 [34]. The landscape of Niterói is more vulnerable to temperature variations and the exposure of immature Ae. aegypti to very high temperatures in small water containers has been attributed as one of the environmental factors leading to slower and heterogeneous wMel invasion. In Rio de Janeiro, Brazil, wMel-infected adults were released into two residential areas between August 2017 and March 2020. At the end of the monitoring period, the wMel invasion and spread to the local Ae. aegypti populations was found to be heterogeneous, and the overall infection rate was 50–70% in the first site and 30–60% in the other site [35]. Releases of wMel Ae. aegypti into two small communities in Nha Trang City in central Vietnam resulted in a seasonal heterogeneity of wMel invasion and spread into the local Ae. aegypti populations with a reduced prevalence of Wolbachia infection in mosquitoes during the hot dry season, followed by an increased prevalence during the cooler season, and such seasonal variation in Wolbachia infection prevalence in mosquitoes was associated with elevated temperature and was possibly due to imperfect maternal transmission of Wolbachia [36]. These studies suggested that the maternal transmission of the two Wolbachia strains can become unstable in Ae. aegypti at high temperatures and is likely to tend to recover back with optimum temperature conditions. Hence, it is important to better understand various factors affecting invasion dynamics of the Wolbachia strains in different settings and seasons to optimise the release strategies.

Long-term studies showed that despite its susceptibility to heat stress, wMel strain has established and persisted in the field at a high frequency within the Ae. aegypti population in many locations in Cairns, Australia, and dengue transmission declined to zero in the release areas [37]. It has been reported that wMel infection has remained stable so far in terms of virus blockage [13] and its effects on fitness [38]. Cairns, Australia, has a tropical climate. The average annual maximum temperature was 29 °C with 62% humidity. During summer, the average temperature ranged from 23.6 °C to 31.4 °C. On rare occasions, the daytime temperature in summer reached 36 °C to 40 °C. In a recent field study in Australia, Ross et al. [39] reported that heat stress on wMel infection had only temporary effects on Wolbachia frequency and
density once the infection had been established in nature. In November 2018, Cairns, Australia, experienced a heatwave of 43.6 °C; subsequently, a sharp decline in the frequency and density of *Wolbachia* was observed in the field population of *Ae. aegypti*, but recovered back closer to 100% 4 months later.

The climate of India comprises a wide range of weather conditions across a vast geographic scale and topography. There are seven climatic regions in India starting from tropical desert to mountain Climate. In most parts of the country, temperature tends to exceed 40 °C during summer months (April–June). Data on water temperature in various types of larval habitats prevalent in these regions during summer are not available, although observations in the simulated studies indicate there could be a difference (lower) of 1–3 °C from the ambient temperature. Considering the climatic conditions in various parts of India, field releases of *Ae. aegypti* mosquitoes transinfected with *Wolbachia* strains should be undertaken during the seasons except the summer months, i.e., from April to June, so that the *Wolbachia* strains will become established among the wild population without undergoing any heat stress.

### Conclusions

The success of *Wolbachia* release programs depends on the stability of *Wolbachia* strains in nature. Monitoring directly under natural conditions is important to assess the effects of heat stress on *Wolbachia* strains. Therefore, pilot field releases need to be undertaken to generate evidence on the stability of the *wMel* and *wAlbB*-infected *Ae. aegypti* (Pud) lines and their thermal tolerance/sensitivity and finally to select a suitable strain for field release in Indian conditions.

### Abbreviations

Pud: Puducherry; Aus: Australia; WMP: World Mosquito Program.

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### Author contributions

PJ and SKS conceptualized the study. KG and CS realised the laboratory and semi-field studies. DP, SD and MK performed molecular testing of the samples. Data analyses were performed by BV. The first draft of the manuscript was written by CS, KG and PJ. AK and MR supervised the study. KG, PJ, SKS and AK revised the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

Supporting data for the conclusion of this article are included within the article. The raw data used for and analysed during this study are available upon reasonable request.

### Declarations

#### Ethical approval and consent to participate

Not applicable.

#### Consent for publication

All authors read and approved the final manuscript.

#### Competing interests

The authors declare no conflict of interest.

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