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Wolbachia introgression in Rio de Janeiro remains at sub-optimal levels 30 months after its decline: challenges in the sustainability of *w*Mel interventions for dengue control

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BACKGROUND The deployment of the *Wolbachia w*Mel strain is currently underway in multiple dengue-endemic municipalities across Brazil. The efficacy of this strategy in Rio de Janeiro remains uncertain, primarily due to the difficulty in sustaining high *w*Mel prevalence in regions previously subjected to large-scale releases. A key contributing factor was the routine rotation of insecticides within the framework of Integrated Vector Management (IVM), which led to the use of the larvicide Spinosad for *Aedes* control in urban areas. This compound was associated with a precipitous decline in *Aedes aegypti* mosquitoes, regardless of the *w*Mel infection status. While *w*Mel-uninfected population recovered within weeks, *w*Mel-infected population remained at low levels likely due to the fitness costs imposed by *w*Mel on egg viability.

OBJECTIVES and METHODS Evaluate the updated frequency of *Wolbachia w*Mel strain in neighborhoods that received deployments. Fort that, we assessed the long-term persistence of *w*Mel following its demographic collapse, by performing mosquito sampling across 12 neighbourhoods in Rio de Janeiro, 30 months after mosquito populations crashed.

FINDINGS Our findings reveal that *w*Mel introgression remains suboptimal, with a mean frequency of 9.87% across the sampled areas. Only two neighbourhoods exhibited *w*Mel frequencies exceeding 15%, likely reflecting ongoing localised releases.

MAIN CONCLUSIONS The reduced prevalence underscores the challenges of achieving self-sustaining *w*Mel establishment in complex urban environments and highlight critical considerations for the implementation of *Wolbachia*-based dengue control programs in endemic regions.

Key words: *Aedes aegypti* - *Wolbachia* - *w*Mel - arboviruses control - dengue - insecticide resistance - integrative vector management

Aedes aegypti (Diptera: Culicidae) is a mosquito species extensively distributed across urban areas of tropical and subtropical regions. It constitutes a major public health concern as the principal vector of several medically important arboviruses, including dengue, Zika, and Chikungunya viruses. Among these, dengue virus (DENV) is particularly significant due to its widespread global presence and the frequent outbreaks and epidemics it causes, affecting millions of individuals annually.[1,2] The burden of dengue is predominantly concentrated in tropical regions, with Brazil consistently reporting the highest number of cases.[3] In 2024 alone, Brazil registered over 6.6 million suspected dengue cases, marking the highest annual incidence since the virus was reintroduced into the country in the late 1980s - early 1990s.[4]

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The continued rise in arboviral cases globally, and particularly in Brazil, demonstrates the limited effectiveness of conventional vector control methods, such as chemical insecticides and mechanical interventions.[5,6,7] This scenario underscores the urgent need for complementary strategies to enhance *Ae. aegypti* population control.[8,9] Among several innovative approaches, the use of the symbiotic bacterium *Wolbachia pipientis* has gained attention. While naturally absent in *Ae. aegypti*, *Wolbachia* strains have been successfully transinfected from other insect species into *Ae. aegypti*, and some of them have pathogen-blocking properties.[10,11,12,13,14] The spread of *Wolbachia* strains through arthropod populations is favoured by a combination of efficient maternal transmission, which is dependent on high *Wolbachia* density in female reproductive systems, and their ability to cause cytoplasmic incompatibility (CI), in which uninfected females are at a disadvantage over infected females because they fail to produce offspring when mated with infected males.[15] It is thereby possible to replace local *Ae. aegypti* populations with high susceptibility to arbovirus with *Wolbachia*-infected equivalents that have a reduced potential for pathogen transmission.

The successful introgression of *Wolbachia* into native *Ae. aegypti* populations involves reaching a high prevalence in the target area with long-term stability. Monitoring efforts are essential to evaluate the proportion of mosquitoes carrying *Wolbachia*, thereby assessing the effectiveness and persistence of the introgression. Notably, *Wolbachia* prevalence in *Ae. aegypti* populations can fluctuate over time and often displays a markedly heterogeneous spatial distribution.[16,17,18,19,20] These fluctuations may be driven by several factors, including reduced fitness of *Wolbachia*-infected individuals, insecticide resistance, migration of wild-type mosquitoes, and environmental conditions such as high temperatures, which can particularly affect the stability of the *w*Mel strain of *Wolbachia*.[21-31]

In the last ten years, *Wolbachia* deployments have been conducted in several cities worldwide from countries as diverse as Australia, Brazil, Colombia, Indonesia, Malaysia, Singapore, and Vietnam, with reports of epidemiological impact.[16,32-39] An intriguing pattern can be observed in Brazil: the neighbouring cities of Rio de Janeiro and Niteroi, although separated each other by a bridge, seems to present a very different introgression profile and epidemiological outcome. In Niterói, *w*Mel mosquitoes were deployed to the full extension of the city limits, which the authors reported to be associated with a substantial reduction of 69% of dengue incidence rate compared to predefined control areas.[40] The outcomes in Rio de Janeiro were more modest and are reported in two manuscripts, with a summary schematic view with the timeline of *w*Mel deployment and monitoring in Fig. 1. The first large-scale deployment in Rio de Janeiro was conducted between January 2017 and December 2019. Despite releasing 67 million *w*Mel *Ae. aegypti* mosquitoes during 2017-2019, an average of only 32% *w*Mel introgression level into the wild population was achieved, which the authors reported to be associated with a reduction of 38% and 10% in dengue and chikungunya notifications, respectively.[41] A second monitoring period was carried out in a subset of the intervention area between August 2021 and December 2023. More than 50,000 mosquitoes were captured in 12 neighbourhoods of Rio de Janeiro, and 7,613 *Ae. aegypti* females were individually screened for *w*Mel presence. In May 2022, a significant decline in *w*Mel frequency was observed across all the 12 neighbourhoods.[42] Authors hypothesised the drop in *w*Mel frequency was associated with the introduction of the larvicide Spinosad (Natular™, Clarke Mosquito Control Products, St. Charles, USA), which disrupted both *w*Mel-infected and -uninfected populations but only the latter was able to recover after an initial population crash likely due to fitness costs of *w*Mel on *Ae. aegypti* mosquitoes.[29,30,43]

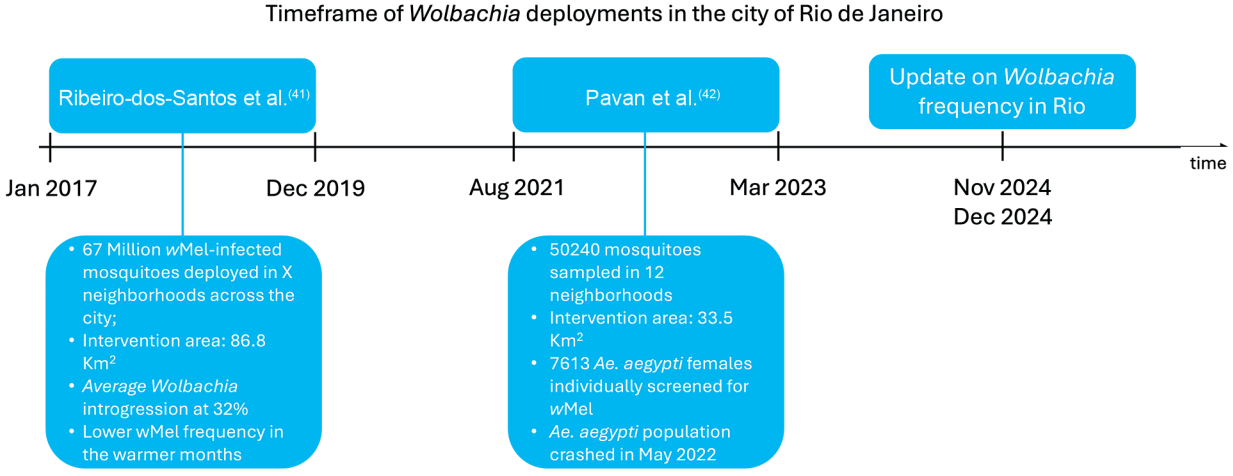


Fig. 1: timeline of *w*Mel deployment and monitoring in the city of Rio de Janeiro, with critical information available in the two previous periods: Jan/2017 to Dec/2019[41] and Aug/2021 to Mar/2023.[42] The *Aedes aegypti* population crashed in May 2022, likely due to an increasing coverage of Spinosad larvicide in the study area.[42]

In this article, we aim to update *Wolbachia* introgression in Rio de Janeiro, 30 months after *Ae aegypti* population crashed in the city, and 20 months after the last observation.[42] By doing so, we plan to provide a snapshot of the current bacteria introgression status in Rio de Janeiro, shedding light on the long-term sustainability of this strategy to mitigate dengue transmission in endemic settings.

MATERIALS AND METHODS

Study area - Field collections of adult mosquitoes were conducted in the same neighbourhoods of Rio de Janeiro as in the longitudinal sampling performed between August 2021 and March 2023.[42] The total area covers 12 urban neighbourhoods: six of them received previous *w*Mel deployment between 2017-2019 from the World Mosquito Program (hereafter named as neighbourhoods A-F) and other six did not (named as G-L) (Fig. 2). This region covers an area of 33.5 km² (38.6% of the WMP release area) where approximately 495,000 people live (55.6% of the population under *w*Mel intervention), encompassing some of the most neglected communities in Rio due to urban violence.

Mosquito sampling - Between November-December 2024, we installed 75 BG-Sentinel traps in the same 12 neighbourhoods and dwellings sampled in Pavan et al.[42] Mosquito sampling was conducted in the same dwellings as Pavan et al.[42] to avoid any bias by comparing *Wolbachia* frequency on different periods. We investigated whether any changes in *w*Mel frequency occurred 30 months after the crash in *Ae. aegypti* populations were observed. The traps were visited weekly until a predefined number of five *Ae. aegypti* females per trap were obtained. Captured mosquitoes were morphologically identified through taxonomic keys[44] and individually screened for the presence of *Wolbachia w*Mel and relative density.

In silico design and validation of oligonucleotides (primers) sets - *Wolbachia* density was estimated through quantitative polymerase chain reaction (qPCR) using specific primers that were designed in this study for SYBR Green-based real-time PCR and amplify 115-bp of the *Wolbachia* surface protein gene (*wsp*; GenBank gene ID CP090948) of *w*Mel and 106-bp of the *Ae. aegypti* ribosomal protein S6 gene (*rps6*; GenBank gene ID 5563590). Briefly, genomic sequences were obtained from the Bacterial and Viral Bioinformatics Resource

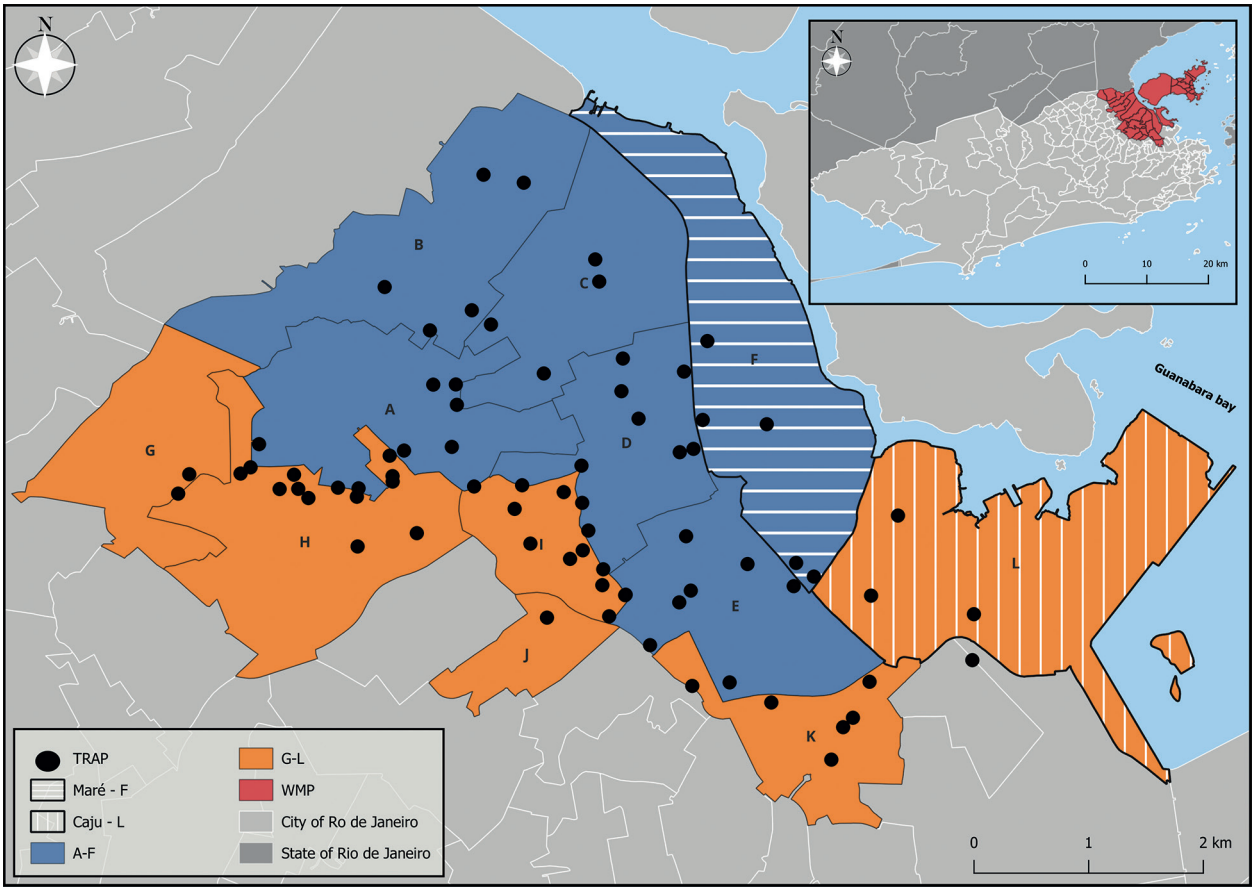


Fig. 2: map of the city of Rio de Janeiro showing in red the total area where mosquito releases were conducted by the World Mosquito Program (WMP). Neighbourhoods included in the study are highlighted in blue (release areas; A: Complexo do Alemão; B: Olaria; C: Ramos; D: Bonsucesso; E: Manguinhos; F: Maré) and orange (non-release areas: G: Engenho da Rainha; H: Inhaúma; I: Higienópolis; J: Maria da Graça; K: Benfica; L: Caju). BG-Sentinel trap locations are marked with black dots. The neighbourhoods of Maré and Caju are indicated with horizontal and vertical hatching, respectively.

Centre v. 3.49 (https://www.bv-brc.org/) and NCBI (https://ncbi.nlm.nih.gov/) databases and sequences were aligned with the MultAlin tool available at http://multalin.toulouse.inra.fr/multalin/. We used *wsp* gene sequences of different *Wolbachia* strains (*w*Mel, *w*AlbA and *w*AlbB) aiming to select a region that would allow specific amplification of the *w*Mel strain. In the case of the *rps* gene, primers were designed in this highly conserved region of *Ae. aegypti*. Primer design was conducted in silico using an online tool (https://primer3.ut.ee/) to generate nucleotide pairs flanking target regions ranging from 50 to 150 base pairs. Finally, the quality of the designed oligonucleotides was evaluated using an online platform specifically developed for this purpose (https://www.idtdna.com). Primers were designed based on key thermodynamic parameters to ensure the specificity and efficiency of qPCR amplification. We selected primers with melting temperatures (Tm) above 45ºC and the difference between the Tm of forward and reverse primers lower than 5ºC. Additionally, the Gibbs free energy (ΔG) was calculated to assess the propensity for the formation of stable secondary structures, which the selected nucleotides had ΔG > -3 kcal/mol and ΔG > -10 kcal/mol to form hairpins and dimers.

The primers originally described by Ross et al.[45] for *rps* presented a very low ΔG, favouring the formation of dimers. The new primers for *rps* are entirely located in the third exon of the *RpS6* gene, ensuring that amplification occurs exclusively in the transcribed regions, increasing efficiency and specificity. Comparative analyses indicate that the *RpS6* gene is highly conserved between *Ae. aegypti* and *Ae. albopictus*, which suggests that the same primers may be applicable to both species. Regarding the primer pairs for *w*Mel, the forward primer was designed in a region highly conserved among the *w*Mel, *w*AlbA and *w*AlbB strains, presenting only three specific nucleotides that differentiate *w*Mel from *w*AlbA. On the other hand, the reverse primer demonstrates greater specificity for *w*Mel, containing ten nucleotides unique to this strain and eight nucleotides shared between *w*Mel and *w*AlbA. Detailed information regarding the designed oligonucleotides is provided in Table.

Laboratory-reared *Ae. aegypti* mosquitoes infected with *w*Mel (F5 generation from Tubiacanga, Rio de Janeiro, Brazil) and not infected with *w*Mel (Paea strain) were used to test primer efficiency and specificity. Mosquitoes were reared in an incubator (Solab model SL-200/364) from egg to adult stage, under a photoperiod of 12 h of light and dark (LD 12:12) and at 28ºC. Females aged 0 to 3 days were flash-frozen in liquid nitrogen. Subsequently, each individual body was homogenised using a pestle, and the resulting material was stored in 350 µL of Buffer RLT Plus (reagent of the Allprep DNA/RNA Mini Kit extraction kit, Qiagen, Hilden, Germany) in a -80ºC freezer until subsequent extraction of genetic material. Total DNA and RNA were extracted using the Allprep DNA/RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol and the DNA was used in the primer testing assays. The concentration of the extracted DNA was determined using the Qubit 4 Fluorometer quantification system (Thermo Fisher Scientific, Waltham, USA).

TABLE

Overview of the specific oligonucleotides data. Details of designed oligonucleotides, including their names, accession IDs, genomic positions, target organisms, and nucleotide sequences

Gene target	GenBank accession (gene ID)	Genome	Organism	Oligonucleotides	Temperature de melting (Tm) (ºC)	Position (nt)
rpS6	5563590	AaeL_AAEL000032	*Ae. aegypti*	aedesRpS6_F_1 (5'-GTGTCCGTCTGCTGCTGAAGA -3') aedesRpS6_R_1 (5'-GACAGGTTCTGGTCAACGATGC -3')	F: 59,5ºC R: 58.2ºC	Primer forward-9005-9025 Primer reverse-9088-9110
wsp	CP090948	546165.19 / *Wolbachia* endosymbiont of *Aedes aegypti* wMel_GV_13	*w*Mel, *w*AlbA, *w*AlbB	wmelwAlbAwsp_F_1 (5'-ATGTAACTCCAGAAGTCAAACTTTATG -3') wmelwsp_R_2 (5'- GCATCAGCAGCCTGTCCG-3')	F: 53,5ºC R: 59.3ºC	Primer forward- 1023582-1023609 Primer reverse- 1023679-1023697

In grey: exon; green: regions conserved among *w*Mel, *w*AlbA, and *w*AlbB; in blue: regions shared between *w*Mel and *w*AlbA; in yellow: regions unique to *w*Mel.

A pool of ten individuals (each individual sample initially had ~10 ng/µL of DNA) of each positive (*w*Mel-infected) and negative (*Wolbachia*-free mosquitoes) control was used to calculate oligonucleotide efficiency and specificity through serial dilutions ranging from 1/1x to 1/32x (12.5-400-ng total DNA). The experiments were performed in a QuantStudio 6, using 7.5 µL of Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), with 0.75 µL of each oligonucleotide at 10 mM, 4 µL of DNA, and 2 µL of water in the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA), under the following regime: an initial incubation at 50ºC for 2 min, followed by an initial denaturation at 95ºC for 10 min. The amplification protocol consisted of 40 cycles of denaturation at 95ºC for 15 s and annealing/extension at 60ºC for 1 min. A melt curve analysis was subsequently performed under the following conditions: 95ºC for 15 s, 60ºC for 1 min, and a gradual increase to 95ºC for 15 s. All reactions were done with three independent replicates.

DNA extraction and Wolbachia quantification in field sampled mosquitoes - Individual females of *Ae. aegypti* were massed in L15 culture medium (Leibovitz) in a Tissue Lyser II apparatus (Qiagen, Hilden, Germany) for 60 s at 50 Hz and the DNA was extracted using the NucleoSpin®Tissue extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. The DNA was quantified in a Qubit 4 Fluorometer quantification system (Thermo Fisher Scientific, Waltham, USA). The relative quantification of *w*Mel in whole mosquitoes was performed with quantitative PCR by amplifying the *Wolbachia wsp* gene, using the *rps6* of *Ae. aegypti* as an endogenous control.[46] Quantitative PCR was performed following the same parameters, reagents, equipment and were run under the same cycling conditions as previously outlined for the primers test. The highest resulting delta threshold cycle (ΔCt) was used as a reference sample for delta delta Ct calculations (ΔΔCt).[47]

Statistical analysis - Descriptive analysis considered three time points to represent the spatiotemporal fluctuation of *w*Mel in Rio de Janeiro. The first two time points used the raw data available in Pavan et al.[42] We selected November/2021 to represent the beginning of the mosquito season in the city and when *w*Mel frequency peaked. We selected July/2022, a few months after the population crashed (May/2022), and sampling was restored to similar numbers as to before the crash. The last time point represents the most recent sampling, in Nov/2024, 30 months after the population crash.

The relative frequency of *w*Mel was compared after the classification of neighbourhoods according to the presence/absence of previous *Wolbachia* deployments. We fitted a generalised linear model (GLM) with a negative binomial distribution with the number of *Wolbachia*-infected mosquitoes per trap as the depended variable and the occurrence of releases as the explanatory variable. In addition, we included a log-transformed offset of the total number of mosquitoes tested pert trap, allowing for the interpretation of the model coefficients in terms of relative rates. Model fit was examined by checking heteroscedasticity, residuals dispersion and the presence of outliers with the R package DHARMa.[48] To test the significance of the predictor variable, we used the type II analysis of deviance. These analyses were performed in the R environment (version 3.6.2).[49]

Assuming *Wolbachia*-mediated arbovirus blocking effect is often density dependent[50] we also quantified the endosymbiont density relatively to the *rps6* of *Ae. aegypti* in field-caught mosquitoes. The *w*Mel density was compared between females captured in areas that received or not *Wolbachia* deployments, as well as with a Fiocruz lab colony using the Kruskal-Wallis test, followed by Dunn multiple comparisons post hoc tests.

Geographic information and choropleth maps - We recorded and stored all entomologic field-derived data, such as the number of trapped and tested adult mosquitoes, along with geocoded address of traps and the neighbourhood, and the *Wolbachia* screening status per sample, in a single database.[25] The maps were produced in Quantum GIS version 3.10.2 (QGIS, https://www.qgis.org). We created choropleth maps to help visualise the *Wolbachia* frequency in sampled *Ae. aegypti* mosquito population among the 12 areas of the city. We built the choropleth maps by dividing the *w*Mel frequencies in five intervals: 0.1-20%; 20.1-40%; 40.1-60%; 60.1-80%; 80.1%-100%.

RESULTS

Primer evaluation - Melting curve analysis revealed a single peak for the *wsp* gene, suggesting that only one fragment is amplified per reaction (Fig. 3). The reaction efficiency for the oligonucleotides targeting the *wsp* gene of *w*Mel falls within the optimal range, with 92.3% efficiency. Regarding the *Ae. aegypti rps6* gene, amplification occurred in both *w*Mel-infected and *Wolbachia*-free mosquitoes (Fig. 4), and the melting curve also presented a single peak in both mosquito groups. The efficiency of the primers was 103.0%, also falling into the optimal range.

Frequency of Wolbachia wMel in field Ae. aegypti - In total, we tested 375 *Ae. aegypti* females from 12 neighbourhoods over the period in which traps remained in the field. There were no significant differences on the number of *Wolbachia*-infected mosquitoes according to the occurrence of previous *Wolbachia* deployments. The coefficient for this explanatory variable was negative but not statistically significant (estimate = -0.3514, z = -0.752, p = 0.452). This finding was supported by the Type II analysis of deviance ($\chi^2 = 0.568$, df = 1, p = 0.451), indicating a similar frequency of *w*Mel mosquitoes in areas adjacent to release zones (Fig. 4), as previously reported (Pavan et al.[42]).

The frequency of *w*Mel in release and non-release areas did not change much between the period of the population crash and the subsequent collection. In March 2023, when the monitoring in Pavan et al. ended,[42] the *Wolbachia* frequencies in release and non-released areas were 13.2% and 5.01%, respectively. In November 2024, the frequency of *w*Mel in released areas dropped to 8%, whereas in the non-release areas it increased to 12% (Fig. 5A). Additional information regarding *w*Mel frequency is available if we aggregate data by neighbour-

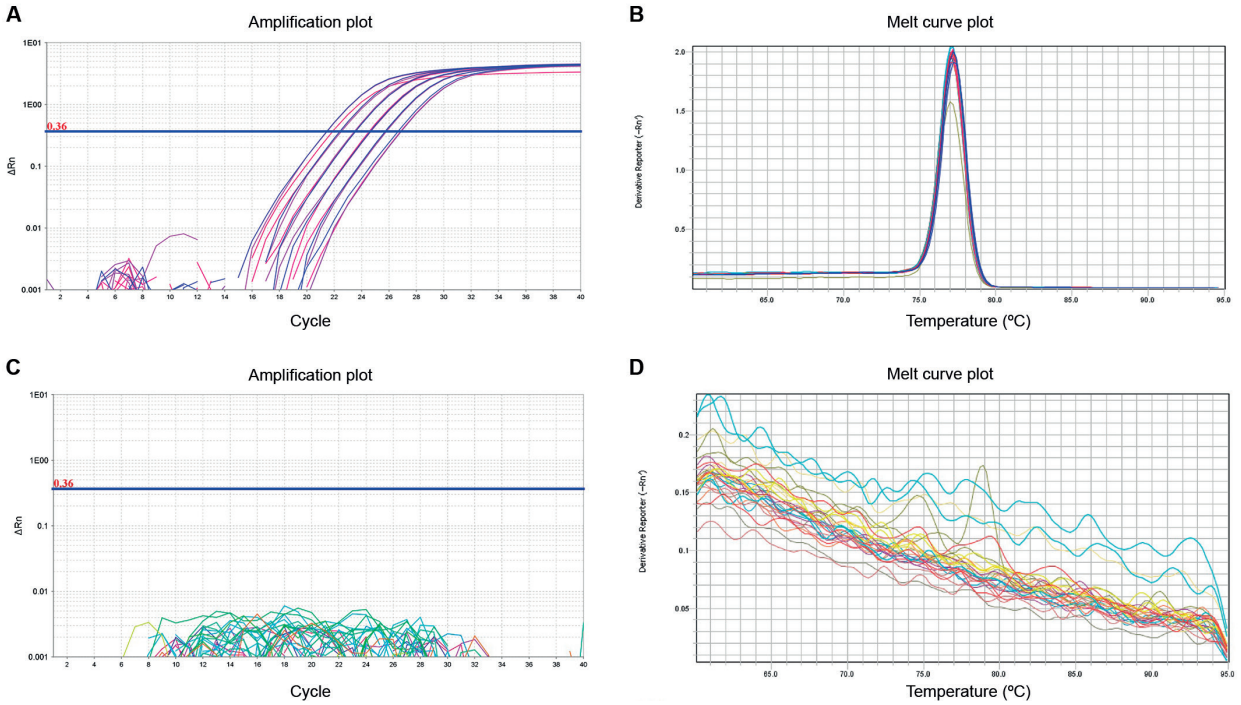


Fig. 3: oligonucleotides for the *wsp* gene of *w*Mel - (A) Amplification curve for the *wsp* gene of *Wolbachia w*Mel, using DNA from *Aedes aegypti* females with *w*Mel; (B) Melting curve for the *wsp* gene of *Wolbachia w*Mel, using DNA from *Ae. aegypti* females infected with *w*Mel; (C) Amplification curve for the *wsp* gene of *w*Mel, using DNA from *Wolbachia*-free *Ae. aegypti* females (*PAEA* strain); (D) Melting curve for the *wsp* gene of *Wolbachia w*Mel, using DNA from *Wolbachia*-free *Ae. aegypti* females (*PAEA* strain).

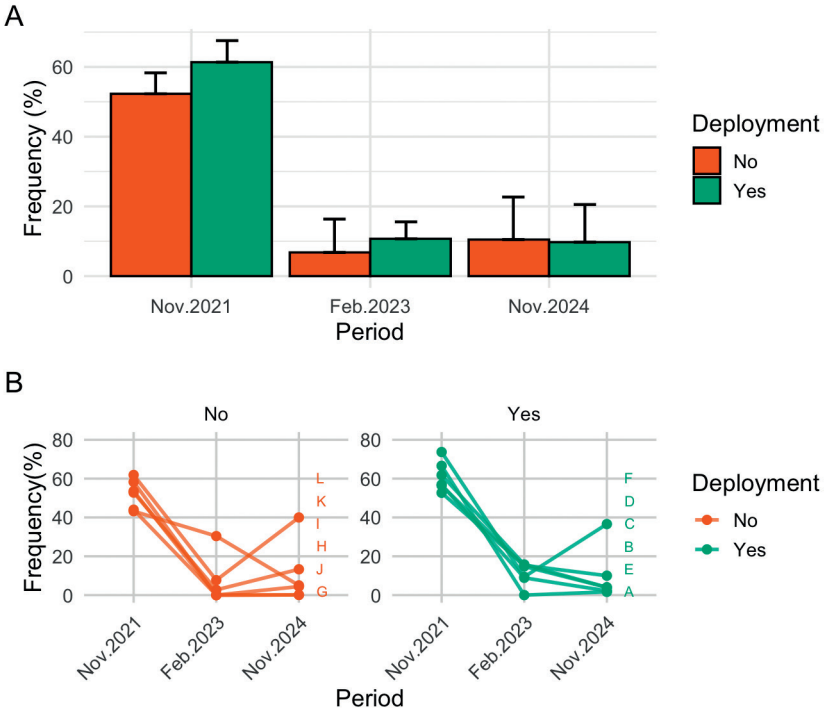


Fig. 4: frequency of mosquitoes (out of five) detected with the *w*Mel strain of *Wolbachia* per trap in neighbourhoods with and without previous mosquito releases by the World Mosquito Program (WMP).

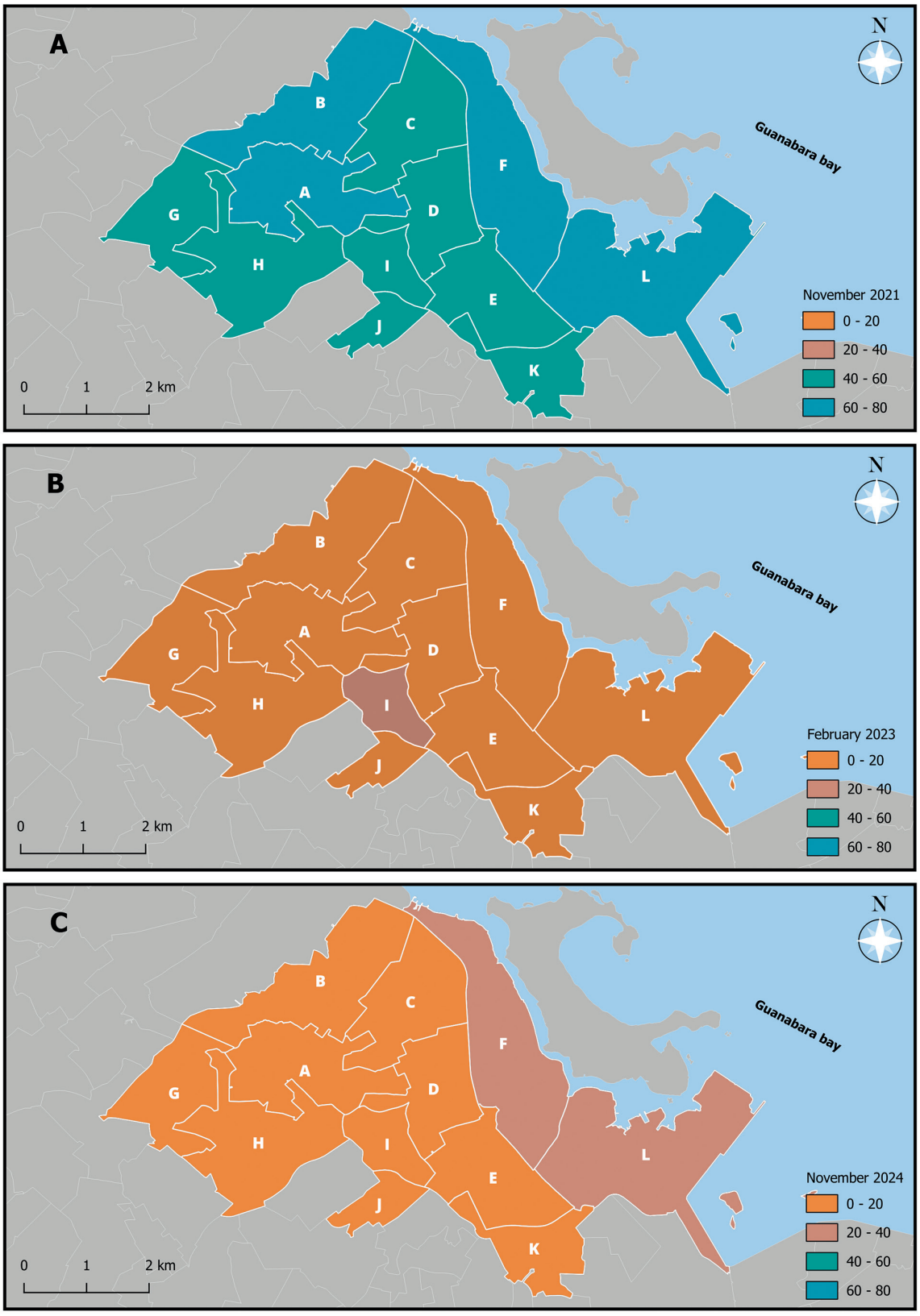


Fig. 5: frequency of *Wolbachia* in female *Aedes aegypti* from the field covering a total period of 3 years, using the raw data from Pavan et al.[42] at November 2021 and July 2022. In light blue (2023 data) and blue (2024 data) are the average frequencies of *w*Mel in neighbourhoods which received *Wolbachia* deployments; in light orange (2023 data) and orange (2024 data) are the frequencies of *w*Mel in areas with no previous *Wolbachia* releases. (A) *w*Mel frequency of neighbourhoods with and without previous releases. (B) Data presented per neighbourhood. Notably, Engenho da Rainha and Maria da Graça had no *w*Mel-infected *Ae. aegypti* captured in both periods.

hood. Considering the neighbourhoods with previous releases (A-F), the *w*Mel frequency was reduced between March 2003 and November 2024, with the exception of the area F. Regarding the neighbourhoods in which no *w*Mel deployment was carried out, in the areas G and J no *Wolbachia*-infected mosquitoes were captured in both evaluation periods. However, a significant increase in *w*Mel frequency was observed in areas K and L, both located in the southern limit of our study area (Fig. 5B). Remarkably, four neighbourhoods had no *w*Mel mosquitoes captured in the BG-Sentinel traps by the end of March 2023. Of these, one had previous releases (A), and three others were without previous deployments (G, H, J). On November 2024, the A and H sites had a frequency of 1.54% and 4.44% of *w*Mel-infected mosquitoes, respectively, whereas sites G and J remained without *w*Mel-infected mosquitoes (Fig. 5B).

The density of the *w*Mel strain in *Ae. aegypti* mosquitoes was consistent between those individuals caught in areas with or without previous deployment, but those from the lab colony had a higher *Wolbachia* density than the ones collected in neighbourhoods that had no releases ($H = 11.23$, $df = 2$, $p = 0.003$, followed by Dunn: $Z = 18.28$, $p < 0.05$)(Fig. 6).

DISCUSSION

The deployment of *Ae. aegypti* carrying *W. pipientis* to mitigate dengue transmission has been underway in different epidemiological settings. In Australia, the *w*Mel strain of *Wolbachia* successfully established in local *Ae. aegypti* populations, reaching fixation and maintaining high prevalence accompanied by a 96% reduction in dengue incidence.[18,51] Similarly, in Yogyakarta, areas achieving > 70% *Wolbachia* prevalence reported a 77% reduction in dengue cases and an 86.2% decline in dengue-related hospitalisations.[52] The deployment of *w*AlbB in Malaysia resulted in an initial average 40% reduction of dengue cases in the intervention sites, rising to > 60% afterwards.[34,35,53] The release of *w*Mel strain in Brazil had divergent patterns in the neighbouring cities of Rio de Janeiro and Niterói for reasons that remain unclear.

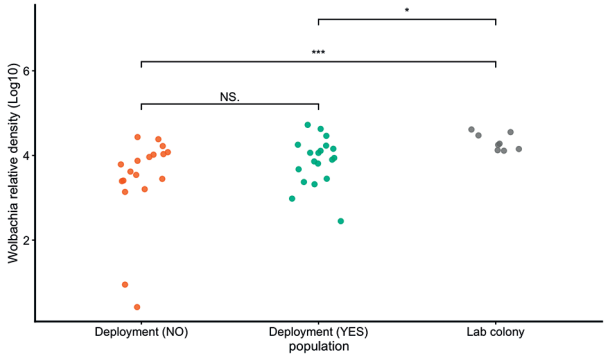


Fig. 6: relative density of *Wolbachia* in *Aedes aegypti* females from the *w*Mel colony and from the field, captured in BG-Sentinel traps installed in areas with or without previous *Wolbachia* releases. The *wsp* gene was used as a target and the *rps6* gene as an endogenous normaliser. Each symbol represents one single mosquito female.

In Niterói, despite heterogeneous introgression patterns across landscapes, an overall 69% reduction in dengue transmission was obtained,[40] while in Rio de Janeiro, a fluctuating *w*Mel prevalence around 32% led to modest reductions in dengue (38%) and chikungunya (10%) incidence.[41] Considering the epidemiological outcome of *Wolbachia* seems to be tightly connected with the level of introgression achieved and the maintenance of high frequency over time, the spatiotemporal evaluation of *Wolbachia* is of utmost relevance to determine its success.

The World Mosquito Program released 67 million *Ae. aegypti* mosquitoes to cover approximately 6.9% of Rio de Janeiro city's area between August 2017 and December 2019.[41] From August 2021 to March 2023 an independent monitoring was able to follow significant changes in *w*Mel frequency.[42] The *w*Mel frequency remained relatively stable between 30-40% until June 2022, when a sharp decline in both *w*Mel-infected and uninfected *Ae. aegypti* populations was observed in Rio de Janeiro. This coincided with the introduction of the new larvicide Spinosad, to which mosquitoes showed full susceptibility. Most likely, the fitness cost associated with this *Wolbachia* strain on mosquito life-history traits prevented its recovery after the Spinosad application coverage was reduced by the local public health office.[28,30,31,42,54-57] Our monitoring revealed that in March 2023 only ~10% of field-caught mosquitoes had *w*Mel. In the present study, we investigated the current profile of *w*Mel introgression in Rio de Janeiro to evaluate potential changes in *Wolbachia* dynamics after its collapse as reported elsewhere.[42] *Wolbachia* frequency remained at low levels similarly to those observed in Pavan et al.[42] The persistence of *w*Mel frequency at ~10% suggest its stabilisation over the last couple of years in Rio de Janeiro. Considering the reproductive advantage conferred by *Wolbachia* is frequency-dependent, its persistence at the low frequencies reported herein will likely be insufficient to trigger a further increase without needing additional releases. Most importantly, there are concerns whether *Wolbachia* frequency at this level can still be effective for protecting against dengue outbreaks.[15,58]

The introduction of the *w*Mel strain of *Wolbachia* into native *Ae. aegypti* populations has been proposed as a promising and sustainable strategy for arbovirus control, based on the assumption that, once a high prevalence threshold is surpassed, *Wolbachia* frequencies remain stable over time.[59,60,61] However, the long-term persistence of *Wolbachia* in field populations appears to be highly dependent on a range of ecological and anthropogenic pressures, including insecticide exposure and climatic variability. In Brazil, the initial field release of *w*Mel-infected *Ae. aegypti* occurred in the geographically isolated fishing community of Tubiacanga, Rio de Janeiro. Although early invasion dynamics were promising, *Wolbachia* frequencies declined sharply following the cessation of releases, coinciding with intensive household level applications of pyrethroid-based adulticides.[16,62] A more extensive collapse in *w*Mel prevalence was observed across a broader urban area, likely associated with the expanded use of the larvicide Spinosad.[41] Similar patterns of instability have been documented

elsewhere; for example, in Tri Nguyen village, Vietnam, *w*Mel frequencies, which had surpassed 90% during cooler periods, dropped to approximately 24% during the hot-dry season, suggesting a pronounced sensitivity to seasonal thermal stress.[20] In Rio de Janeiro, seasonal oscillations in *Wolbachia* prevalence have likewise been recorded, with the lowest frequencies observed during the summer months — coinciding with the peak incidence of dengue transmission.[41] As in Tri Nguyen village, results in Rio de Janeiro also showed small-scale heterogeneity, but most likely due to additional releases conducted by the WMP.[63] The observation that only the neighbourhoods of Maré (area F) and Caju (area L) exhibited an increase in *w*Mel frequency is likely attributable to successive rounds of mosquito releases, as reported in local media sources.[63] The necessity of supplementary releases in areas where *Wolbachia* frequencies remain below optimal thresholds should be considered an integral and ongoing component of vector control programs, particularly in light of the environmental factors that can influence *w*Mel dynamics. Additional local boost releases to overcome micro-geographic drops on *Wolbachia* frequency must be done when routine surveillance detects poor introgression in areas where deployment has taken place. Furthermore, given the historical role of Rio de Janeiro as a major port of entry for both dengue serotypes and genotypes, ensuring that *w*Mel frequencies remain at protective levels in previously targeted areas should take precedence over the expansion of releases into new localities.[64,65] The prevalence threshold of *w*Mel to effectively protect local communities is still unknown, but it must consider the *Wolbachia* strain, the spatiotemporal heterogeneity of its frequency, herd immunity, and dengue serotype, to mention a few. A preliminary result showed that introgression < 45% was not associated with a reduced dengue incidence during 2024 in Rio de Janeiro city.[66] However, further investigations are required to understand whether this threshold can be extrapolated elsewhere.

An additional consequence of suboptimal *w*Mel frequencies in the field is the reduced level of community protection, as lower prevalence of *Wolbachia* is expected to result in diminished interruption of arbovirus transmission. The epidemiological consequences of *Wolbachia* deployment are unlikely to be uniform across transmission settings and should be interpreted in light of local arbovirus dynamics. Rather than fully blocking dengue transmission, the introduction of the *w*Mel strain is generally expected to reduce the effective reproductive number (R_0). Modelling studies suggest that when transmission intensity is low to moderate, such reductions may be sufficient to interrupt transmission. However, in highly endemic contexts the same effect may translate primarily into decreases in incidence rather than complete suppression of outbreaks.[67] This distinction is particularly relevant for settings such as Rio de Janeiro, where dengue transmission can reach very high levels during epidemic years. Notably, in 2024, Rio de Janeiro experienced its most severe dengue outbreak in the past decade.[68] Clearly, the reported increase in dengue transmission is not directly related to the drop in *Wolbachia* frequency given it covers less than 7% of the city's total area. However, spatiotemporal Bayesian models were fit to estimate the effect of introgression on dengue incidence during 2020-2024.[67] Authors observed the *Wolbachia* deployment was not associated with reductions in dengue incidence in 2024, amid a large dengue epidemic was reported in Rio de Janeiro city.[67] Moreover, introgression < 45% was not associated with reduced dengue incidence.[67] The updated frequency of *w*Mel in Rio de Janeiro revealed all 12 neighbourhoods sampled had introgression levels below the 45% threshold suggested.[67]

The *w*Mel density of field-caught mosquitoes from the area without previous releases was significantly lower than the lab colony, following the same pattern as observed before.[43] As extensively reported, high temperatures can negatively impact *w*Mel density in *Ae. aegypti*.[23,26,27] Considering lab mosquitoes are reared in optimum insectary conditions, with a stable temperature of 27 ± 2ºC, *Wolbachia* density of lab colony mosquitoes is expected to be high. Regarding arbovirus mitigation, it is well known that the pathogen-blocking phenotype associated with *Wolbachia* depends on its density.[69,70,71] Therefore, seasonal fluctuations in *w*Mel density may affect *w*Mel effectiveness, especially if a decrease is observed during the warmer periods in which dengue transmission is naturally more intense.

This study has limitations. The sampling strategy encompassed only a subset of the *w*Mel deployment area, thereby constraining the ability to generalise findings to non-monitored regions. Additionally, BG-Sentinel traps were preferentially installed in the same households as those used in Pavan et al.,[42] which facilitates temporal comparison but may perpetuate any sampling biases present in the original dataset. Despite these constraints, the study offers a robust and independent assessment of the spatiotemporal distribution of the *w*Mel strain in Rio de Janeiro. These findings highlight the critical importance of adopting an Integrated Vector Management (IVM) framework to enhance the efficiency and sustainability of vector control efforts and disease prevention strategies and to engage in ongoing monitoring and adaptative releases when deploying *Wolbachia*.

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AUTHORS' CONTRIBUTION

Conceptualisation - MGP, FJG, GAG, OGC, DV and RMF; data curation - FJG, MRD, FO, LPB and LD; formal analysis - MRD, JCA, KPP, GAG, FO, OGC and DV; investigation - MGP, GAG, AJM, VC, JBPL, GLW, AH, OGC, DV and RMF; methodology - MGP, FJG, JCA, KPP, FO, LPB, LD, OGC and DV; project administration and resources - MGP, DV and RMF; supervision - MGP, AJM, JBPL, AH, DV and RMF; visualisation - JCA, KPP, LPB, LD, OGC and DV; writing - original draft - MRD, MGP, OGC, DV and RMF; writing - review & editing - MGP, FJG, MRD, JCA, KPP, GAG, FO, LPB, LD, AJM, VC, JBPL, GLW, AH, OGC, DV and RMF. The authors declare no conflict of interests.

DATA AVAILABILITY

After publication the data will be available on demand to corresponding author.

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OPEN PEER REVIEW

Memórias do IOC thanks the anonymous reviewers for their contribution to the peer review of this work.

FIRST REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

Overall Assessment

This study examines the persistence of Wolbachia wMel in Aedes aegypti in Rio de Janeiro 30 months after a major population collapse. The topic is highly relevant to dengue control, and the longitudinal monitoring effort is commendable. However, the manuscript is difficult to follow in places, lacks key data presentation (notably spatial maps), and makes confident claims that are not always well supported by evidence. Methodological choices, particularly the introduction of a new molecular assay without proper validation, further complicate interpretation. Substantial revision is needed before the study can be considered for publication

Major Comments

1) Spatial presentation of results

• The study is explicitly about neighborhood-level heterogeneity in wMel persistence, yet spatial information is only presented in tables and bar charts. Without maps or spatial analyses, it is very hard for the reader to discern geographic patterns or continuity of areas with low prevalence.

• Spatial mapping would considerably strengthen the manuscript and should be included. At present, the narrative feels fragmented and unconvincing.

2) New molecular assay and comparability over time

• A new TaqMan qPCR assay was developed, justified only by a claim that the prior assay could form primer dimers. However, no validation data are provided (e.g. sensitivity, specificity, reproducibility, or comparison with the established method).

• Because temporal comparisons are central to the manuscript, using different assays across years without cross-validation undermines confidence in the claim of declining wMel prevalence.

• At minimum, authors should present validation data or side-by-side comparisons to demonstrate assay equivalence. Without this, their conclusions risk being artefactual.

3) Sample size and statistical robustness

• The study tested only 375 females across 12 neighborhoods (~39% of the release area?). This is a limited dataset for such strong claims.

• Please provide confidence intervals around prevalence estimates and clarify the statistical power.

• The GLM model is mentioned but not well described (covariates, diagnostics, rationale for negative binomial). Supplementary materials should include model diagnostics.

4) Attribution of Wolbachia loss to Spinosad

• The manuscript strongly attributes the decline in wMel to spinosad larvicide use. While plausible, this is a correlative association. Other drivers (climate variability, immigration of uninfected mosquitoes, stochastic drift) are not fully considered. The discussion should be more balanced and avoid over-interpretation.

5) Interpretation of epidemiological impact

• The link to Rio's 2024 dengue epidemic is tenuous, as Wolbachia releases covered <7% of the city. The text should avoid implying causation at this scale. The current framing is overly confident and risks overstating public health impact.

6) Figures and clarity

• Figure 5: Barplots of Wolbachia prevalence lack confidence intervals. Given small sample sizes, CIs (e.g., binomial or Wilson) are essential to show uncertainty. Without them, differences may appear more robust than they are.

• I assume that in Figure 6: "NO Wolbachia" means individuals without Wolbachia infection. Yet, this group is shown with positive values for the loads. This is confusing. The authors must clarify how infection status was defined, why density values are shown for negatives, and whether these represent assay noise, sub-threshold infections, or plotting artefacts.

• qPCR output plots currently in the main figures could be moved to Supplementary Data, with only summary results retained. This would streamline the main text.

7) Programmatic implications

• The conclusion that Wolbachia must be "reinforced before expanding elsewhere" is sensible, but currently too general. What prevalence threshold is considered protective? Explicitly linking findings to WHO guidelines or programmatic targets would increase the utility of the recommendations.

Minor Comments

1) Abstract: rephrase “Regardless the wMel infection status” → “Regardless of the wMel infection status.”

2) Introduction: More clearly connect the cited heterogeneity studies (refs 16–20) to Rio’s unique context.

3) Methods: Provide more detail on neighborhood selection and improve Figure 1 map.

4) Results: Present neighborhoods with zero wMel in tabular form for clarity.

5) Discussion: Expand the point on seasonal thermal stress and its likely contribution to Wolbachia instability in Rio.

Recommendation: Major revision.

The study is potentially valuable but is currently weakened by limited spatial presentation, lack of molecular assay validation, and overconfident causal claims. Addressing these issues with additional data, more cautious interpretation, and clearer figures will be necessary for publication.

REVIEWER #2

This paper examining Wolbachia establishment in Rio de Janeiro for dengue control is a little confusing in its framing and the conclusions it draws from very limited data. The general narrative is that Wolbachia has somehow been established and then lost from mosquito populations in Rio and this is somehow connected to the ecological perturbation of using Spinosad insecticides. Everything is presented as correlation without demonstration of causality. I think a much simpler explanation for the observed data is that Wolbachia establishment never succeeded in Rio initially. Successful introgression requires the Wolbachia frequency to exceed the unstable point as defined by the models of Michael Turelli. The reported Wolbachia frequencies in Rio – around 30-40% are very close to the measured unstable point of approx. 0.3 as measured in Cairns, Australia for the wMel strain. Given this closeness I think a more likely and simpler explanation than invasion followed by Wolbachia loss is that invasion was never accomplished (as defined by robustly exceeding the unstable point). Turelli’s models predict that the closer the Wolbachia frequency is to the unstable point the slower the introgression dynamics will be – either for invasion or loss and indeed what appears to be happening in Rio is a gradual loss of infection, consistent with the modelling, and this loss is happening slowly as predicted. The issue of the Spinosad insecticide application having some causal link with this loss seems highly unlikely as both populations of Wolbachia infected and uninfected mosquitoes are expected to be equally susceptible to the insecticide and so it should not be important in influencing the introgression dynamics. So, in my opinion the authors are trying to weave a complex narrative around something that is much simpler, and that is that Wolbachia was never successfully established and is slowly decaying in frequency in these areas. There is no need to invoke more complicated explanations and in fact this could be quite misleading. The key point as mentioned by authors is that in adjoining neighbourhoods in Niteroi, the introgression is complete and stable and experiences the same weather conditions as Rio, arguing against a general climate issue affecting introgression. Interestingly, when releases were first undertaken in Jurajuba, in Niteroi, there was initial difficulty in establishing Wolbachia, requiring re-releases but once established it was extremely robust.

So, while the paper attempts to make the claim that complex environments might lead to loss of Wolbachia a better conclusion in my opinion is that complex environments may make establishment of Wolbachia more challenging. Indeed, in this setting there were poorer neighbourhoods that routinely experience elevated levels of violence, interrupting release activities for weeks at a time during deployment. These disruptions together with possible underdosing may be much more likely to explain the introgression dynamics. While initial dose can be an important consideration, we also need to consider the role of the unhatched egg bank in certain settings which will influence establishment dynamics and could be an important but relatively unexplored critical element of the mosquito ecology that will influence Wolbachia establishment. Indeed, it may have been a critical element explaining the dynamics of Wolbachia in Jurajuba, Niteroi. Considering the novelty of the intervention I think it is normal that as we undertake deployments at increasing scale in a Public Health context we can expect some failures and optimisation that will be required in learning the best ways to deploy. However, that is very different from thinking that the method is inherently unstable. The success in Niteroi is evidence against the latter conclusion.

AUTHORS’ RESPONSE TO THE REVIEWERS

REVIEWER COMMENTS:

Reviewer: 1

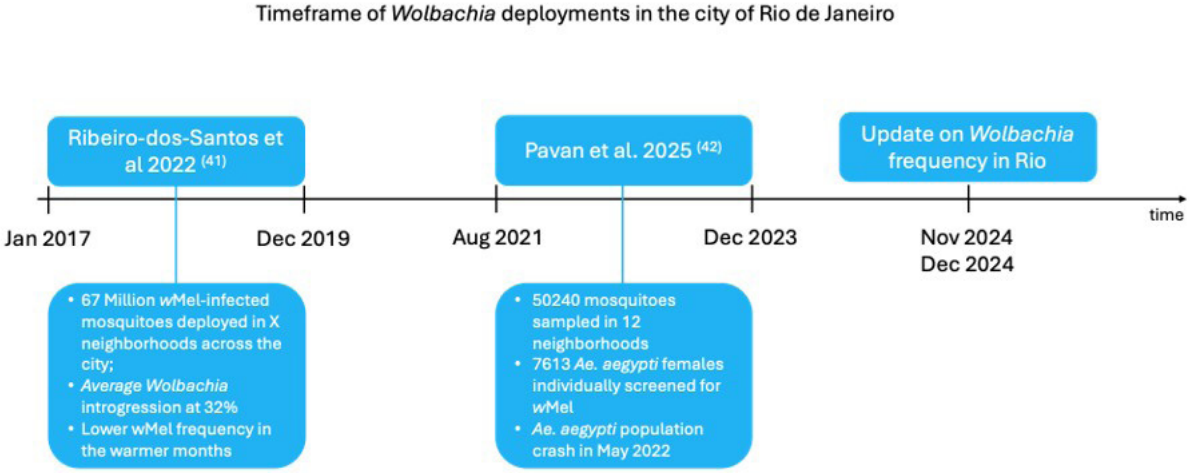
Overall Assessment

This study examines the persistence of Wolbachia wMel in Aedes aegypti in Rio de Janeiro 30 months after a major population collapse. The topic is highly relevant to dengue control, and the longitudinal monitoring effort

is commendable. However, the manuscript is difficult to follow in places, lacks key data presentation (notably spatial maps), and makes confident claims that are not always well supported by evidence. Methodological choices, particularly the introduction of a new molecular assay without proper validation, further complicate interpretation. Substantial revision is needed before the study can be considered for publication.

We appreciate Reviewer observations, although we believe most of the key data requested is available in the publication in which we demonstrated the collapse of *Wolbachia* after individually screening by RT-qPCR wMel presence in 7,613 field-collected *Ae. aegypti* (Pavan et al. 2025). The aim of the manuscript under evaluation is to share an update of *w*Mel frequency exactly 30 months after it crashed due to an increase in coverage of the larvicide Spinosad in the study area.

We acknowledge the fact this manuscript is an update of the actual *Wolbachia* frequency was not properly described. Therefore, we edited several sections of the manuscript to make it clearer in the second version, and created a new Figure with a timeline of Rio de Janeiro large-scale deployments, from 2019-2025 (became Figure 1 in the manuscript, as it is below). The other points raised will be discussed in the following comments.



Major Comments

1) Spatial presentation of results

• The study is explicitly about neighborhood-level heterogeneity in wMel persistence, yet spatial information is only presented in tables and bar charts. Without maps or spatial analyses, it is very hard for the reader to discern geographic patterns or continuity of areas with low prevalence.

• Spatial mapping would considerably strengthen the manuscript and should be included. At present, the narrative feels fragmented and unconvincing.

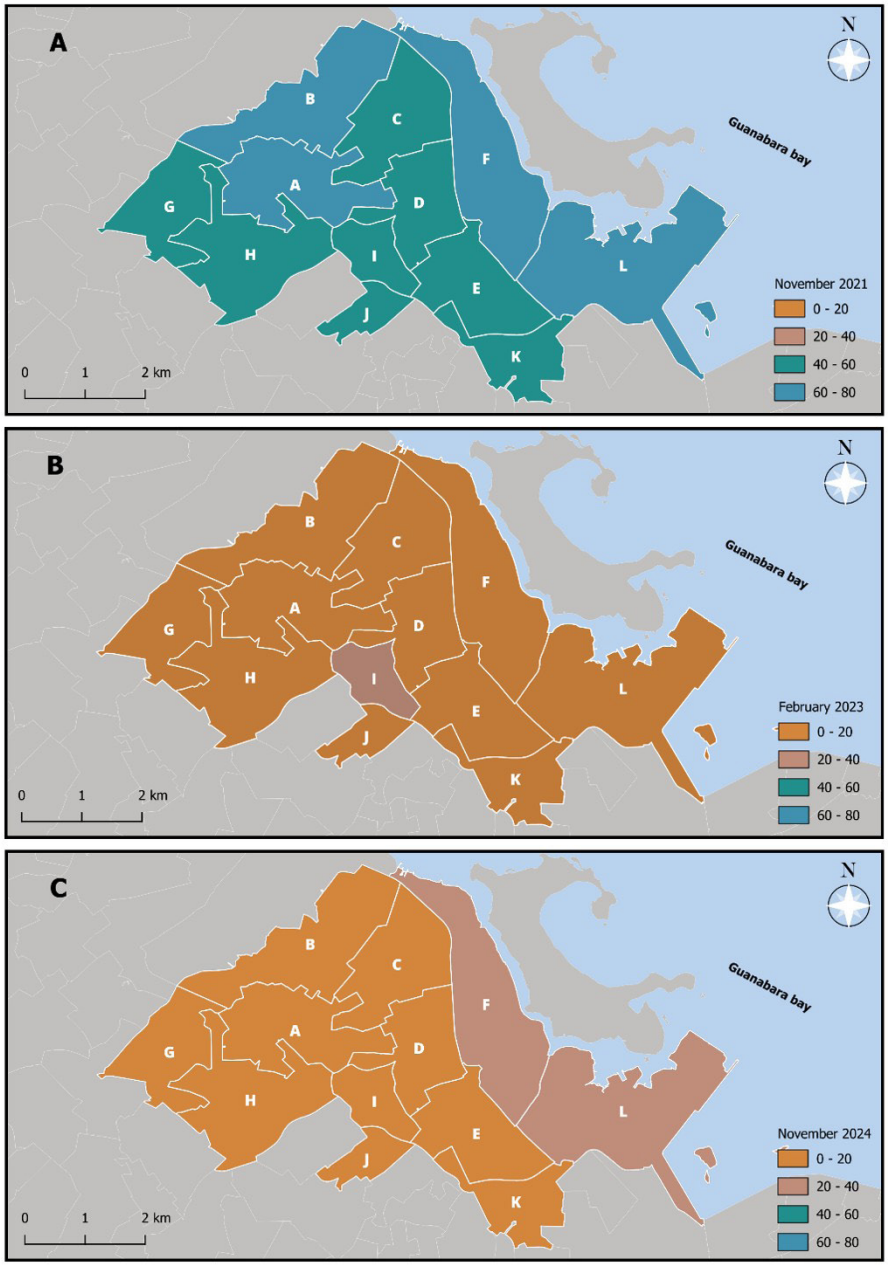
We appreciate Reviewer observation regarding the spatial information of *w*Mel persistence. As mentioned now in the manuscript, we selected three representative periods of the time series from Aug/2021 to Dec/2024:

November 2021 to represent the beginning of the mosquito season in the city and when wMel frequency peaked.

July/2022, a few months after the population crashed in May/2022, and sampling was restored to similar numbers as to before the crash.

Nov/2024 The last time point represents the most recent sampling, exactly 30 months after the population crash.

We did choropleth maps to show the spatiotemporal variations on *Wolbachia* frequency during the timeframe of three full years, from November 2021-2024. The maps are now listed as Figure 5 and are presented below.



2) New molecular assay and comparability over time

• A new TaqMan qPCR assay was developed, justified only by a claim that the prior assay could form primer dimers. However, no validation data are provided (e.g. sensitivity, specificity, reproducibility, or comparison with the established method).

• Because temporal comparisons are central to the manuscript, using different assays across years without cross-validation undermines confidence in the claim of declining wMel prevalence.

• At minimum, authors should present validation data or side-by-side comparisons to demonstrate assay equivalence. Without this, their conclusions risk being artefactual.

We appreciate the reviewer's concern, but *Wolbachia* was screened in Pavan et al. (2025) through conventional PCR. Therefore, since we made qPCR assays, this new method is more sensitive than the one used previously. This way, we probably detected *Wolbachia*-positive samples even when *Wolbachia* load was below the limit of detection of a conventional PCR.

We believe, however, that we could added more information on these new primer pairs. As previously explained in the Methods section, the new primers were validated in silico to ensure high specificity and efficiency of qPCR amplifications. Moreover, experimental validation steps were performed, such as the calculation of primer

efficiency, melting curve, and analysis of amplification plots of serial dilutions of samples. In this new version, we also added information on the limit of quantification of primer pairs, and calculations of coefficient of variation to determine their specificity and reproducibility (lines 220-226, and 294-298).

3) Sample size and statistical robustness

• The study tested only 375 females across 12 neighborhoods (~39% of the release area?). This is a limited dataset for such strong claims.

We believe our dataset is sufficient to provide an update of the Wolbachia frequency 30 months after its collapse in Rio de Janeiro, described in Pavan et al. (2025). Just to reinforce, in Pavan et al. (2025), the collapse was identified after establishing a longitudial sampling of 20 monthis in which 19,437 *Ae. aegypti* females were sampled and 7,613 individually screened for *Wolbachia*. In this manuscript, we returned to the same 75 collection points and updated the *w*Mel frequency after collecteg 5 females per trap, i.e., 375 individuals.

• Please provide confidence intervals around prevalence estimates and clarify the statistical power.

We have provided the confidence intervals over prevalence estimates.

• The GLM model is mentioned but not well described (covariates, diagnostics, rationale for negative binomial). Supplementary materials should include model diagnostics.

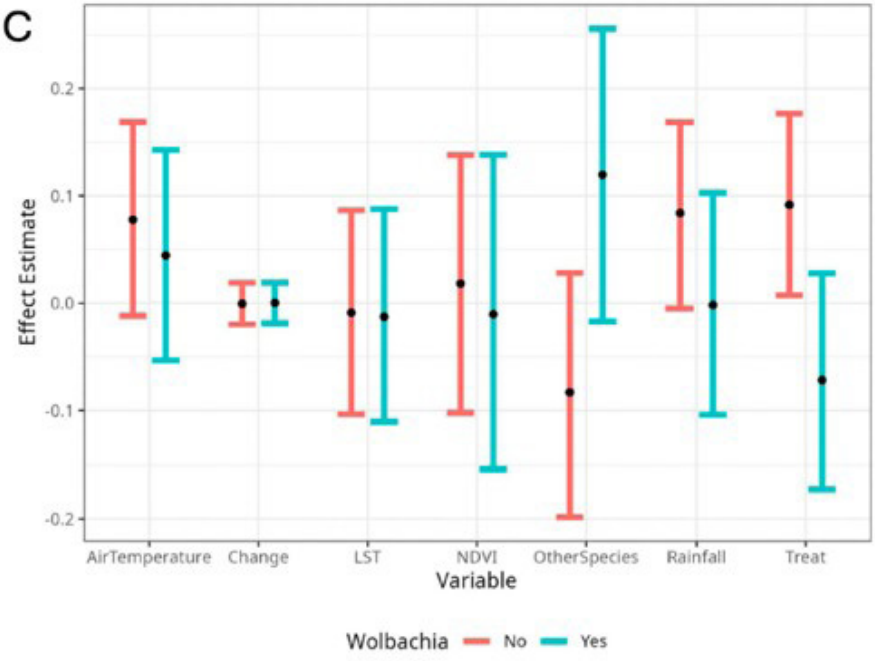
These analysis details were included in the methods section (lines 234-249), as well as model diagnosis plots are now available in Supplementary material.

4) Attribution of Wolbachia loss to spinosad

• The manuscript strongly attributes the decline in wMel to spinosad larvicide use. While plausible, this is a correlative association. Other drivers (climate variability, immigration of uninfected mosquitoes, stochastic drift) are not fully considered. The discussion should be more balanced and avoid over-interpretation.

We believe there is misunderstanding regarding a possible claim in this manuscript that *Wolbachia* loss was caused by Spinosade. We actually showed it in another paper (Pavan et al. 2025, ref #42 of the cited references). At Pavan et al. 2025, we conducted a 20-month longitudinal investigation and sampled 50,240 mosquitoes, i.e., the claim that Spinosade caused the *Wolbachia* loss was based on a much higher sampling effort. At this manuscript, we conducted a new round of mosquito sampling in the same 75 locations as reported in Pavan et al. (2025). I.e., the sampling was conducted over the same 12 neighborhoods to update the *w*Mel frequency at Rio de Janeiro 30 months after *Wolbachia* crashed (as explained in details at Pavan et al. 2025).

Still, we agree with the reviewer when he/she mentions that other drivers (climate variability, immigration of uninfected mosquitoes, stochastic drift) could influence *Wolbachia* loss. Again, in the paper Pavan et al. 2025 (ref #42), we tested the impact of external variables - air temperature, change to spinosad (lagged by 4 months), land temperature (LST), NDVI, presence of other mosquitoes, rainfall - as inferred from the MCMC simulations, see below.



Full data is available at figshare: https://doi.org/10.6084/m9.figshare.27477987.v1 allowing further analysis. But looking for all the evidence available and data analysis, we hypothesized the increase in Spinosad coverage reduced mosquito population, both *Wolbachia*-infected and -uninfected, in the study area, but only the latter could recover when larvicide application ceased due to a well-known fitness cost in egg hatching due to *Wolbachia* presence.

5) Interpretation of epidemiological impact

• The link to Rio's 2024 dengue epidemic is tenuous, as Wolbachia releases covered 7 of the city. The text should avoid implying causation at this scale. The current framing is overly confident and risks overstating public health impact.

We appreciate Reviewer observation. We have edited different sections of the Discussion to avoid cause-effect relations between dengue epidemic in 2024 and the drop in Wolbachia frequency. The most critical section now reads: "An additional consequence of suboptimal *w*Mel frequencies in the field is the reduced level of community protection, as lower prevalence of *Wolbachia* is expected to result in diminished interruption of arbovirus transmission. Notably, in 2024, Rio de Janeiro experienced its most severe dengue outbreak in the past decade. [66] Clearly, the reported increase in dengue transmission is not directly related to the drop in *Wolbachia* frequency given it covers less than 7% of the city's total area. However, spatiotemporal Bayesian models were fit to estimate the effect of introgression on dengue incidence during 2020-2024. [67] Authors observed the *Wolbachia* deployment was not associated with reductions in dengue incidence in 2024, amid a large dengue epidemic was reported in Rio de Janeiro city. [67] Moreover, introgression >45% was associated with increased dengue incidence. [67] The updated frequency of *w*Mel in Rio de Janeiro revealed all 12 neighborhoods sampled had introgression levels below the 45% threshold suggested. [67]"

6) Figures and clarity

• Figure 5: Barplots of Wolbachia prevalence lack confidence intervals. Given small sample sizes, CIs (e.g., binomial or Wilson) are essential to show uncertainty. Without them, differences may appear more robust than they are.

We agree with Reviewer recomendation and we have produced new graphs, more informative. As we stated above, we selected three timepoints covering a total of three years of monitoring:

November 2021 to represent the beginning of the mosquito season in the city and when wMel frequency peaked.

February/2023, to represent an intermediate point of the timeseries considering its 36-month range.

Nov/2024 The last time point represents the most recent sampling, exactly 30 months after the population crash.

We reformulated the figures to better represent the spatiotemporal fluctuation in the wMel frequency observed. We adopted error bars in the new Figure 4A.

• I assume that in Figure 6: "NO Wolbachia" means individuals without Wolbachia infection. Yet, this group is shown with positive values for the loads. This is confusing. The authors must clarify how infection status was defined, why density values are shown for negatives, and whether these represent assay noise, sub-threshold infections, or plotting artefacts.

We have edited the figure.

• qPCR output plots currently in the main figures could be moved to Supplementary Data, with only summary results retained. This would streamline the main text.

We agree with reviewer recommendation and moved the qPCR plots to supplementary data.

7) Programmatic implications

• The conclusion that Wolbachia must be "reinforced before expanding elsewhere" is sensible, but currently too general. What prevalence threshold is considered protective? Explicitly linking findings to WHO guidelines or programmatic targets would increase the utility of the recommendations.

We agree with Reviewer in regards of the prevalence threshold that is considered protective. However, there is currently strong debate to define it. The work presented under reference #67 fitted spatiotemporal Bayesian models to estimate the effect of introgression on dengue incidence during 2020-2024. Authors observed strong association between dengue incidence and wMel introgression below 45%. We have added the following sentences to the Discussion section: "The prevalence threshold of *w*Mel to effectively protect local communities is still unknown, but it must consider the *Wolbachia* strain, the spatiotemporal heterogeneity of its frequency, herd immunity, and dengue serotype, to mention a few. A preliminary result showed that introgression <45% was associated with increased dengue incidence during 2024 in Rio de Janeiro city. [67] However, further investigations are required to understand whether this threshold can be extrapolated elsewhere."

Minor Comments

1) Abstract: rephrase "Regardless the wMel infection status" → "Regardless of the wMel infection status."

2) Introduction: More clearly connect the cited heterogeneity studies (refs 16–20) to Rio's unique context.

3) Methods: Provide more detail on neighborhood selection and improve Figure 1 map.

4) Results: Present neighborhoods with zero wMel in tabular form for clarity.
5) Discussion: Expand the point on seasonal thermal stress and its likely contribution to Wolbachia instability in Rio.

Done as recommended.

Recommendation: Major revision.

The study is potentially valuable but is currently weakened by limited spatial presentation, lack of molecular assay validation, and overconfident causal claims. Addressing these issues with additional data, more cautious interpretation, and clearer figures will be necessary for publication.

We appreciate the Reviewer comments on the first version of this manuscript. We hope the edited version has solved the concerns raised.

Reviewer: 2

This paper examining Wolbachia establishment in Rio de Janeiro for dengue control is a little confusing in its framing and the conclusions it draws from very limited data. The general narrative is that Wolbachia has somehow been established and then lost from mosquito populations in Rio and this is somehow connected to the ecological perturbation of using Spinosad insecticides. Everything is presented as correlation without demonstration of causality. I think a much simpler explanation for the observed data is that Wolbachia establishment never succeeded in Rio initially. Successful introgression requires the Wolbachia frequency to exceed the unstable point as defined by the models of Michael Turelli. The reported Wolbachia frequencies in Rio – around 30-40% are very close to the measured unstable point of approx. 0.3 as measured in Cairns, Australia for the wMel strain. Given this closeness I think a more likely and simpler explanation than invasion followed by Wolbachia loss is that invasion was never accomplished (as defined by robustly exceeding the unstable point). Turelli's models predict that the closer the Wolbachia frequency is to the unstable point the slower the introgression dynamics will be – either for invasion or loss and indeed what appears to be happening in Rio is a gradual loss of infection, consistent with the modelling, and this loss is happening slowly as predicted. The issue of the Spinosad insecticide application having some causal link with this loss seems highly unlikely as both populations of Wolbachia infected and uninfected mosquitoes are expected to be equally susceptible to the insecticide and so it should not be important in influencing the introgression dynamics. So, in my opinion the authors are trying to weave a complex narrative around something that is much simpler, and that is that Wolbachia was never successfully established and is slowly decaying in frequency in these areas. There is no need to invoke more complicated explanations and in fact this could be quite misleading. The key point as mentioned by authors is that in adjoining neighbourhoods in Niteroi, the introgression is complete and stable and experiences the same weather conditions as Rio, arguing against a general climate issue affecting introgression. Interestingly, when releases were first undertaken in Jurajuba, in Niteroi, there was initial difficulty in establishing Wolbachia, requiring re-releases but once established it was extremely robust.

So, while the paper attempts to make the claim that complex environments might lead to loss of Wolbachia a better conclusion in my opinion is that complex environments may make establishment of Wolbachia more challenging. Indeed, in this setting there were poorer neighbourhoods that routinely experience elevated levels of violence, interrupting release activities for weeks at a time during deployment. These disruptions together with possible underdosing may be much more likely to explain the introgression dynamics. While initial dose can be an important consideration, we also need to consider the role of the unhatched egg bank in certain settings which will influence establishment dynamics and could be an important but relatively unexplored critical element of the mosquito ecology that will influence Wolbachia establishment. Indeed, it may have been a critical element explaining the dynamics of Wolbachia in Jurajuba, Niteroi. Considering the novelty of the intervention I think it is normal that as we undertake deployments at increasing scale in a Public Health context we can expect some failures and optimisation that will be required in learning the best ways to deploy. However, that is very different from thinking that the method is inherently unstable. The success in Niteroi is evidence against the latter conclusion.

We appreciate Reviewer observations. Although we believe most of the key data requested is available in the publication in which we demonstrated the collapse of *Wolbachia* after individually screening by RT-qPCR wMel presence in 7,613 field-collected *Ae. aegypti* (Pavan et al. 2025). The aim of the manuscript under evaluation is to share an update of *w*Mel frequency exactly 30 months after it crashed due to an increase in coverage of the larvicide Spinosad in the study area.

We acknowledge the fact this manuscript is an update of the actual *Wolbachia* frequency was not properly described. Therefore, we edited several sections of the manuscript to make it clearer in the second version, and created a new Figure with a timeline of Rio de Janeiro large-scale deployments, from 2019-2025.

Regarding the very important point raised about whether *Wolbachia* establishment suceeded or not, previous work by Ribeiro-dos-Santos et al. (2022) showed a dengue reduction of 38% and 10% in dengue and chikungunya transmission in the areas with *Wolbachia*. Although it is definitely a lower protective effect than achieved to Niterói, it seems to strong and somehow not fair to mention its introgression has never suceeded. After all, if there is no succesful invasion there should be no protective effect from it. Due to massive literature available from Brazil and other endemic sites, we strongly believe *Wolbachia* worked in Rio but at suboptimal levels. And part of our ongoing research is to understand the reasons why it worked poorly in Rio. The concept that "invasion was never accomplished" pointed ou by reviewer, is controversial. *Wolbachia* deployment ended by the World

Mosquito Program at December 2019 and the wMel strain frequency remained reasonably stable until May 2023. If *Wolbachia* invasion was never accomplished, as stated by Reviewer, how could we explain its frequency maintenance for 3.5 years without additional releases?

Turelli's model has been largely invocated to define *Wolbachia* dynamics into its hosts. However, as any other model, it does not fully represents reality. For instance, the theory underlying the bistable equilibrium states *Wolbachia* should invade or fail. However, we have very compeiling evidence showing the co-existence of *Wolbachia*-infected and -uninfected individuals, both at a smaller spatial scale but also at a larger area, references #16 and #42, respectively. As mentioned before, the increase in Spinosad coversage during the summer of 2022 overlaps with the mosquito season, but this year we had very few mosquito population in the study areas, meaning both *Wolbachia*-infected and -uninfected were affected. The negative effect of *Wolbachia* on egg-hatching is well-documented and it implies the *Wolbachia*-infected mosquitoes do not have an egg bank to recover after a significative suppression in adult population takes place. But this is a story already published in Pavan et al. (2025). The manuscript herein deals only with an update frequency of wMel in the same area of Rio de Janeiro, providing thus a current snapshot about *Wolbachia* frequency.

SECOND REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

I am not satisfied with the response of the authors on my prior comments. My expectation is that this alternative hypothesis which can also explain the observations be included in the discussion as an alternative hypothesis for the data. Wolbachia establishment (meant as introgression that is stable) has not been met and the possible positive epidemiological impact is expected and not relevant to the question of whether the Wolbachia infection is stable or not. I don't think this alternative view can be so easily ignored to support the over dramatised conclusion drawn from the current version.

In relation to the epidemiology it is also notable that a key paper on the proposed impact of Wolbachia on disease impact is missing and I believe would strengthen the paper if it was cited and discussed. Ferguson et al Science Translational Medicine 2015 (DOI: 10.1126/scitranslmed.3010370) shows that the epidemiological impact of Wolbachia is not absolute but represents a reduction in the effective R0 which in situations where the R0 is moderate will lead to elimination, but when the R0 is extremely high as was seen in Brazil in 2024 transmission reduction will not lead to disease elimination, but still produce a drop in disease. The paper would be strengthened by including this nuance in the discussion in relation to the epidemiology.

I also believe the overly dramatic word "crash" in the title is misleading and instead should be replaced with "introduction".

AUTHORS' RESPONSE TO THE REVIEWERS

Reviewer: 1

I am not satisfied with the response of the authors on my prior comments. My expectation is that this alternative hypothesis which can also explain the observations be included in the discussion as an alternative hypothesis for the data. Wolbachia establishment (meant as introgression that is stable) has not been met and the possible positive epidemiological impact is expected and not relevant to the question of whether the Wolbachia infection is stable or not. I don't think this alternative view can be so easily ignored to support the over dramatised conclusion drawn from the current version.

We thank the reviewer for raising this important point and for encouraging consideration of alternative interpretations. However, the temporal dynamics observed in Rio de Janeiro points a much more complex scenario than suggested by mathematical modeling and data from elsewhere (Australia and Southeast Asia). The bistable equilibrium suggested by *Wolbachia* modeling papers (Hoffmann et al. 1990; Barton & Turelli 2011) is challenged by field data that reveals introgression is not a binomial outcome, but an index that fluctuates over space and time, as revealed by a careful observation in public available data from Rio de Janeiro and Niteroi (Brazil: Garcia et al. 2019; Pinto et al. 2021; Ribeiro-dos-Santos et al. 2022). This scenario has been commonly described as "incomplete introgression", as pointed out by authors associated with the World Mosquito Program (Ribeiro-dos-Santos et al. 2022), presenting moderate effect on dengue incidence. Remarkably, full introgression (commonly pointed as >60%) has been observed and associated with strong impact on dengue incidence in Yogyakarta (Indonesia: Utarini et al. 2021), Bello, Medellin and Itaguí (Colômbia: Velez et al. 2023), and Townsville (Australia: O'Neill et al. 2019).

Specifically on Rio de Janeiro. After the completion of wMel releases in December 2019, wMel frequencies remained relatively stable (30-50%) from January 2020 to April 2022, a period comprised of 28 months. From

our perspective, a clear case of "incomplete introgression" as classified by Ribeiro-dos-Santos et al. (2022). Nevertheless, the infection frequency persisted at stable levels for 28 months in the field after the cessation of releases.

Previous mark-release-recapture studies in Rio de Janeiro demonstrated an average life expectancy of female *Aedes aegypti* of ~15 days (Maciel-de-Freitas et al. 2007). Therefore, this period roughly corresponds to around 56 mosquito generations without additional wMel releases. Persistence across such many generations strongly suggests that Wolbachia introgression had reached a stable although incomplete introgression prior to a disturbance that was observed in April 2022.

Regarding the epidemiological outcome of Wolbachia deployment. Notably, previous analyses conducted in Rio de Janeiro (Santos et al. 2022; DOI: 10.1016/S1473-3099(22)00436-4) reported a similar pattern of incomplete introgression, with wMel frequencies remaining around 30–40% between August 2017 and December 2019. Despite this intermediate level of introgression, Wolbachia deployments were associated with a 38% reduction in dengue incidence and a 10% reduction in chikungunya incidence. These findings suggest that even moderate wMel frequencies may contribute to reductions in arbovirus transmission at the population level. A comparable pattern was also observed in Zone 3 of Niterói during the 2017–2019 releases (Pinto et al., 2021; DOI: 10.1371/journal.pntd.0009556). These observations suggest that the scenario observed, characterized by incomplete yet temporally stable wMel introgression persisting for several years after the cessation of releases, may represent a realistic outcome in large and environmentally heterogeneous urban settings. Such dynamics may therefore occur in other Brazilian regions where this technology is currently being implemented or planned and should be considered when evaluating the long-term performance of Wolbachia-based interventions, rather than interpreting intermediate frequencies as a failure of establishment.

For comparison, a contrasting outcome consistent with failure of wMel establishment was reported by Garcia et al. (2019) - DOI: 10.1371/journal.pntd.0007023. Following the first round of wMel releases in Tubiacanga (Rio de Janeiro), wMel frequency peaked at approximately 65% at the end of the release period but declined rapidly to ~10% within 04 months after releases ceased. This rapid decline indicates a lack of persistence of the infection in the field once releases stopped. In contrast, in the present scenario in Rio de Janeiro, wMel frequencies remained relatively stable at approximately 30–40% for about 28 months after the end of releases, indicating a markedly different temporal dynamic.

Regarding discussing hypothesis for the crash observed in Wolbachia wMel strain In Rio, it was exhaustively debated in Pavan et al. (2024). It is also important to clarify that the objective of the present paper is not to investigate the mechanisms responsible for the rapid decline in wMel frequency observed in Rio de Janeiro in April 2022. The description of the longitudinal sampling and the hypothesis in which the *w*Mel crash was due to the high coverage of the biolarvicide Spinosad was explored in the paper published by Pavan et al. (2024). In this manuscript, our aim is solely to provide an update on *Wolbachia* frequency after the end of the monitoring reported in Pavan et al. (2024).

Hoffmann, A. A. , Turelli M., and Harshman L. G.. 1990. Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans* . Genetics 126:933–948.

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In relation to the epidemiology it is also notable that a key paper on the proposed impact of Wolbachia on disease impact is missing and I believe would strengthen the paper if it was cited and discussed. Ferguson et al Science Translational Medicine 2015 (DOI: 10.1126/scitranslmed.3010370) shows that the epidemiological impact of Wolbachia is not absolute but represents a reduction in the effective R0 which in situations where the R0 is moderate will lead to elimination, but when the R0 is extremely high as was seen in Brazil in 2024 transmission reduction will not lead to disease elimination, but still produce a drop in disease. The paper would be strengthened by including this nuance in the discussion in relation to the epidemiology.

We thank the reviewer for this valuable suggestion. We agree that this study provides important context for interpreting the expected epidemiological impact of *Wolbachia* deployments. Following the reviewer's recommendation, we have incorporated the reference Ferguson et al. (2015) into the Discussion and expanded the text to emphasize that *Wolbachia* is expected to reduce the effective reproductive number (R_0), rather than completely block transmission. This point has now been incorporated into the Discussion before addressing the epidemiological patterns observed in Rio de Janeiro.

I also believe the overly dramatic word "crash" in the title is misleading and instead should be replaced with "introduction".

We thank the reviewer for this comment. We have now replaced the word "crash" with "decline" in the title of the manuscript. However, the term "crash" was originally introduced in Pavan et al. (2024), where the authors described and discussed the rapid decline in wMel frequency observed in Rio de Janeiro and explored the potential mechanisms underlying this pattern. Because the present study builds directly upon the scenario described in that previously published work, we opted to maintain the same terminology to ensure consistency with the existing literature.

In addition, the study done by Pavan et al (2024) has already been cited by subsequent publications that refer to the same system and context (e.g., Corrêa-Antônio et al. 2025, DOI: 10.1038/s42003-025-08862-7; Corbel et al. 2025, DOI: 10.1371/journal.pntd.0013675; Anders et al. 2025, DOI: 10.3390/tropicalmed10090237; de Morais Batista et al. 2025, DOI: 10.1016/j.lana.2025.101327). Therefore, rather than sound "over dramatized", we believe that maintaining the terminology helps preserve continuity with the emerging literature discussing Wolbachia dynamics in Brazilian settings, besides preserving the seriousness required in investigations in the field of public health.

THIRD REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

No comments.

DECISION: ACCEPT SUBMISSION | RECEIVED 15 AUGUST 2025 | ACCEPTED 05 MAY 2026