Large-scale deployment and establishment of *Wolbachia* into the *Aedes aegypti* population in Rio de Janeiro, Brazil.

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Abstract

Traditional methods of vector control have proven insufficient to reduce the alarming incidence of Dengue, Zika and chikungunya in endemic countries. The bacterium symbiont *Wolbachia* has emerged as an efficient pathogen-blocking and self-dispersing agent that reduces the vectorial potential of *Aedes aegypti* populations and potentially impairs arboviral disease transmission. In this work, we report the results of a large-scale *Wolbachia* intervention in Ilha do Governador, Rio de Janeiro, Brazil. *w*Mel-infected adults were released across residential areas between August 2017 and March 2020. Over 131 weeks, including release and post-release phases, we monitored the *w*Mel prevalence in field specimens, and analyzed introgression profiles of two assigned intervention areas, RJ1 and RJ2. Our results revealed that *w*Mel successfully invaded both areas, reaching overall infection rates of 50-70% in RJ1, and 30-60% in RJ2 by the end of the monitoring period. At the neighborhood-level, *w*Mel introgression was heterogeneous in both RJ1 and RJ2, with some profiles sustaining a consistent increase in infection rates and others failing to elicit the same. Correlation analysis revealed a weak overall association between RJ1 and RJ2 (r = 0.2849, P = 0.0236), and an association at a higher degree when comparing different deployment strategies, vehicle or backpack-assisted, within RJ1 (r = 0.4676, P < 0.0001) or RJ2 (r = 0.6263, P < 0.0001). The frequency *knockdown resistance* (*kdr*) alleles in *w*Mel-infected specimens from both areas was consistently high over this study. Altogether, these findings corroborate that *w*Mel can be successfully deployed at large-scale as part of vector control intervention strategies, and provide the basis for imminent disease impact studies in Southeastern Brazil.

Introduction

Fighting the mosquito *Aedes aegypti* (= *Stegomyia aegypti*) sounds almost like a mantra for human populations living the tropics, whose lives are constantly threatened by diseases attributed to this species. Dengue (DENV), Zika (ZIKV) and chikungunya (CHIKV) viruses are among the many etiological agents transmitted by *Ae. aegypti*, highlighting its status as a major disease vector (Kraemer et al. 2015; WHO 2017). Global estimates of DENV alone point to around 400 million annual infections (Bhatt et al. 2013), distributed in over 128 countries (Brady et al. 2012). While the largest burden is in Asia (Bhatt et al. 2013), South American countries have long been hit by outbreaks and account for a considerable quota. In Brazil, notified cases of DENV sum up to 1.5 million annually, according to current surveillance reports (SVS 2019; 2021).

Without effective vaccines to tackle arboviral infections, public health authorities rely exclusively on vector control strategies (Thisyakorn and Thisyakorn 2014; Abdelnabi, Neyts, and Delang 2015; Lin et al. 2018). Management of breeding sites and deployment of chemical pesticides are the most common suppression methods, both with serious pitfalls. The former, usually performed by public agents and community members themselves, lacks precision and workforce, as suitable sites are vast in urban landscapes (Valença et al. 2013; Carvalho and Moreira 2017). In addition, *Ae. aegypti* egg loads are difficult to spot and remain viable for many months in nature (Rezende et al. 2008). As for the latter, natural selection of resistant variants has been the real issue (Maciel-de-Freitas et al. 2014; Melo Costa et al. 2020), downplaying the efficacy of current compounds and constantly pushing their replacement by new ones. Thus, innovative strategies tackling these issues and providing a more efficient, sustainable, control over arboviral infection are a welcome addition to traditional approaches in use.

79 One such strategy is the field deployment of *Wolbachia*-infected *Ae. aegypti*.
80 *Wolbachia pipientis* is an obligatory intracellular bacterial endosymbiont, naturally present in
81 around 40% of arthropods (Zug and Hammerstein 2012), which manipulates host
82 reproductive biology to increase its inheritance rates (Werren, Baldo, and Clark 2008). When
83 artificially introduced into *Ae. aegypti*, some *Wolbachia* strains such as *w*Mel or the virulent
84 *w*MelPop were able to trigger cytoplasmic incompatibility (CI) in reciprocal crosses with wild
85 specimens, and rapidly invade confined populations (Walker et al. 2011). In addition, and of
86 particular importance to arboviral disease control, these newly established *Wolbachia*-
87 mosquito associations led to pathogen interference (PI) phenotypes, possibly involving the
88 modulation of immune system (Rancès et al. 2012) and metabolite pathways (i.e. intracellular
89 cholesterol) (Caragata et al. 2014; Geoghegan et al. 2017). *Wolbachia*-harboring *Ae. aegypti*
90 lines have shown refractoriness to infection by DENV, ZIKV, CHIKV and other medically
91 relevant arboviruses (Moreira et al. 2009; Ferguson et al. 2015; Dutra et al. 2016; Aliota,
92 Peinado, et al. 2016; Aliota, Walker, et al. 2016; Pereira et al. 2018; Carrington et al. 2018;
93 Flores et al. 2020). Levels of refractoriness, nonetheless, seem to vary between strains, with a
94 putative tradeoff with fitness costs (Joubert et al. 2016).
95 Supported by promising experimental data, *w*Mel-infected *Ae. aegypti* were used in
96 pioneer field release trials in Northern Australia, promoting the bacterium spread and
97 establishment into natural mosquito populations (A. A. Hoffmann et al. 2011; Ary A.
98 Hoffmann et al. 2014). Importantly, *Wolbachia*'s high prevalence rates in the field, as well as
99 intrinsic CI and PI, were sustained in the long-term, providing the necessary conditions to
100 reduce dengue incidence in subsequent epidemiological assessments (O'Neill et al. 2019;
101 Ryan et al. 2019). Corroborating the Australian findings, recent trials in Indonesia
102 (Tantowijoyo et al. 2020) and Southeastern Brazil (Garcia et al. 2019; Gesto et al. 2020) have
103 also reported the successful invasion and establishment of *w*Mel at some localities, with

preliminary evidence of arboviral disease reduction (Indriani et al. 2020; Durovni et al. 2020; Pinto et al. 2021). In the particular context of Southeastern Brazil, trials have initially targeted small neighborhoods of Rio de Janeiro and the nearby city Niterói, following adult (Garcia et al. 2019) or egg deployment methods (Gesto et al. 2020). With high *w*Mel frequencies, and DENV and ZIKV refractoriness maintained intact over the post-release period (Gesto et al. 2020), additional areas of both cities could be considered for *Wolbachia* implementation.

In this study, we report the results of a large-scale field release of *w*Mel-infected *Ae. aegypti* in Rio de Janeiro, covering all the populated area of Ilha do Governador. We analyze the *Wolbachia* introgression profile, both from an overall and a more detailed neighborhood-specific perspective. To control for known operational risks we assess the *knockdown resistance* (*kdr*) profiling of colony and field specimens during our intervention. Lastly, we compare the outcomes of different adult deployment methods, 'vehicle' or 'backpack', and relate them to different urban and social contexts.

Results and discussion

To evaluate the performance of a large-scale field deployment of *Wolbachia* in Brazil, we targeted the whole urban territory of Ilha do Governador, Rio de Janeiro. Being the largest island of Guanabara bay, with an estimated population of 211,018 and a total area of 40 km^2, Ilha do Governador was an ideal starting point for testing expanded deployment interventions. First, because one of its neighborhoods, Tubiacanga, hosted a successful small trial in recent years (Garcia et al. 2019). And second, because islands are less prone to migration of wild mosquitoes from adjacent areas, which could affect the invasion dynamics. For logistical reasons, Ilha do Governador was divided into two great intervention areas, RJ1 and RJ2, each comprising a subset of neighborhoods (Figure 1). An additional layer was added by allocating

129 sections to different deployment strategies: vehicle (V) or backpack-assisted (B). The former
130 was the preferred choice, delivering speed and coverage, but was limited to areas with proper
131 road organization, in which minivans could circulate and reach release sites. The latter was
132 chosen for community settlements with informal housing and narrow passages, usually
133 associated with favelas (i.e. slums). In this case, release sites could only be reached on foot.

134 *w*Mel-infected *Ae. aegypti* (*w*MelRio) (Garcia et al. 2019) were mass-released across
135 RJ1 and RJ2, following specific schedules for each area (Figure 2, Figure S1, Supplementary
136 Table S1). Mosquito deployments were carried in three rounds with 'resting' periods in-
137 between, from August 2017 to March 2019 in RJ1, and from November 2017 to March 2019
138 in RJ2. To monitor *Wolbachia* presence in the field, BG-Sentinel traps were mounted in
139 suitable households (Figure S2, Supplementary Table S2) and adult *Ae. aegypti* caught were
140 tested weekly/fortnightly for *w*Mel infection. By analyzing the frequency of positive
141 individuals (i.e. prevalence rates) over the 131 weeks spanning the entire release and post-
142 release phases, the introgression of *w*Mel in RJ1 and RJ2, and across Ilha do Governador as a
143 whole, were analysed (Figure 2).

144 *w*Mel introgression in RJ1 was characterized by a steep increase in prevalence rates
145 over the first two release rounds, peaking at 60-80%, and a subsequent and self-sustaining
146 frequency of 50-70%, until the end of monitoring (Figure 2A). At the neighborhood level
147 (Figure 3) *w*Mel introgression was heterogenous in RJ1, suggesting that invasion dynamics
148 were not consistent across the intervention area. In some of the neighborhoods where releases
149 were vehicle-assisted, such as Bancários, Freguesia, Monero, Tauá, Cacuia and Praia da
150 Bandeira the introgression profiles showed consistent increases in *w*Mel prevalence over time,
151 resulting in high prevalence rates (>80%) by March 2020. Others, such as Pitangueiras,
152 Cocotá and Ribeira, had more heterogenous profiles, with alternating mid-high (60-80%) and
153 low (<50%) *w*Mel frequencies over time, with non-consistent trends by the end of March

2020. The neighborhood of Zumbi reached moderately high *w*Mel prevalence (60-70%) following the second round of releases, but monitoring was then suspended in March 2019 due to very low mosquito abundance, precluding further observation. The areas with backpack-assisted releases, aggregated and analyzed as a single unit named RJ1.B, showed a slow and consistent rise in *w*Mel prevalence towards high levels (>80%), suggesting successful wMel introgression (Figure 3).

In RJ2, the overall introgression profile was characterized by oscillating *w*Mel frequencies (30-60%), with prevalence rates increasing over the second and third release rounds but not self-sustaining afterwards (Figure 2B). Once again, individual neighborhood results indicate a complex, spatially variable picture of wMel introgression (Figure 3). Here, in vehicle-assisted release areas, Jardim Guanabara was the best performing, with a classical invasion trend stabilizing at high prevalence rates (~80%). Jardim Carioca and Portuguesa, on the other hand, were less successful and had persistently low frequencies (30-40%). Unlike the two categories above, Galeão and Cidade Universitária had mid-level wMel frequencies (30-60%), similar to the overall RJ2 profile. These two neighborhoods account for most of the territory enclosed in RJ2, with sparse building blocks and a peculiar, mostly non-resident human occupation. Galeão hosts the city's international airport, and Cidade Universitária, as the name suggests, hosts the federal university campus. In backpack-assisted release areas, RJ2.B, prevalence rates also increased during second and third release rounds, but soon after declined to low levels and, at the time of last monitoring in March 2020, did not yet demonstrate evidence of *w*Mel introgression.

Despite intrinsic differences in their overall profiles, RJ1 and RJ2 are still weakly associated in Spearman's correlation analysis ($r = 0.2849$, $P = 0.0236$) (Figure 4A), suggesting that factors underlying invasion are shared at some level between intervention areas. Hence, RJ1 and RJ2 data were aggregated into a single profile reflecting the overall

179 panorama of *Wolbachia*'s invasion in Ilha do Governador (Figure 2C). With prevalence rates
180 ranging from 55 to 65% by the end of field monitoring, this panorama suggests that *w*Mel
181 introgression is still an on-going process in Ilha do Governador. This representation, however,
182 must be understood as an oversimplified indicative of its invasion dynamics, hiding an
183 underlying complexity at the neighborhood (or neighborhood section) level.

184 A similar analysis was undertaken to compare vehicle and backpack-assisted sections,
185 within RJ1 (Figure 4B) and RJ2 contexts (Figure 4C). Spearman's correlation indicates a
186 moderate association between RJ1.V and RJ.B ($r = 0.4676$, $P < 0.0001$), and a strong
187 association between RJ2.V and RJ2.B ($r = 0.6263$, $P < 0.0001$), suggesting that the outcomes
188 of both deployment strategies covary within the same region. Nonetheless, the efficiency of
189 each strategy, which ultimately translates to weekly prevalence rates and invasion trends, was
190 variable between intervention areas and possibly affected by non-controlled events. In
191 backpack-assisted sections, RJ1.B and RJ2.B, release intervention was often impaired by
192 violent drug-related conflicts. During the third round, RJ1.B had 5 weeks of interruption due
193 to this reason alone, and RJ2.B had 6, extending the release period to 17 or 28 weeks,
194 respectively (Supplementary Table S1). Interestingly, should interruptions of this kind
195 influence invasion dynamics, then RJ2.B was certainly more affected, as revealed by our
196 failed attempt to stably introgress *w*Mel by the end of this study period (i.e. week 131).

197 We previously deployed *w*Mel in the small community of Tubiacanga on the Ilha do
198 Governador along 2014 and 2015. Here, *w*Mel initially failed to establish because of
199 mismatched genetic backgrounds between the release mosquito strain and the resident wild-
200 type population, raising particular attention to insecticide resistance-related traits (Garcia et al.
201 2019). It was only after repetitive rounds of backcrossing, with introgression of wild allelic
202 variants, that the *w*Mel-infected line was enough fit to promote a successful invasion. With
203 this in mind, we monitored the genetics of pyrethroid resistance by screening for mutations in

204 the voltage-gated sodium channel (*Na$_v$*), known as *kdr* (knockdown resistance), in field caught
205 *Wolbachia*$^+$ samples from RJ1 and RJ2 (Figure 5). As we could observe, the allelic profiling
206 of samples from both intervention areas revealed the predominance of resistant variants,
207 *Na$_v$R1*(1016Val$^+$, 1534Cys^{kdr}) and *Na$_v$R2* (1016Ile^{kdr}, 1534Cys^{kdr}), and shortage of the
208 susceptible one, *Na$_v$S* (1016Val$^+$, 1534Phe$^+$), corroborating the findings of a nation-wide
209 survey (Melo Costa et al. 2020). Over the monitoring period, this profile experienced little
210 variation within and between areas, indicating the long-term maintenance of *kdr* mutations in
211 *w*Mel-infected field samples, and highlighting its adaptive role in pyrethroid-infested
212 environments. Moreover, it rules out the possibility that the differences observed in invasion
213 trends along this trial could be influenced by *kdr* frequencies.
214 To drive a successful invasion, *Wolbachia* must interact with bacterium-free *Ae.*
215 *aegypti* populations and underlying factors that influence its maintenance and density in the
216 natural habitat (Hancock et al. 2016; Schmidt et al. 2017). Here, especial attention should be
217 given to quiescent egg loads, which are known to remain viable for many months (up to over
218 a year) in the habitat, waiting for favorable conditions to resume. With a reduced resistance to
219 desiccation, *w*Mel-infected eggs are critically impacted by climate and have a significant
220 decay in viability in periods over 40 days (Farnesi et al. 2019). Although it is not clear how
221 much it costs to invasion profiles, it is still an underlying factor to consider when analyzing
222 different contexts. From this perspective, human settlements with fewer inhabitants and/or
223 better management of breeding sites, aided by community participation in vector control
224 surveillance, could be prone to lower *Ae. aegypti* densities and faster, more efficient,
225 *Wolbachia* invasion. In contrast, crowded human settlements and undermined control of
226 breeding sites tend to promote higher *Ae. aegypti* densities and slower, less efficient, invasion
227 dynamics.

228 Even though some individual neighborhoods of Ilha do Governador failed to elicit
229 invasion trends, it is possible that this scenario reverts on its own in the future. Here,
230 migration from adjacent neighborhoods (Schmidt et al. 2017), with higher prevalence rates,
231 may play an important contribution and act synergistically with *w*Mel self-driving ability, as
232 expressed by the CI mechanism. In other words, *Wolbachia* hotspots like Bancários,
233 Freguesia, Monero, Praia da Bandeira, Jardim Guanabara and Tubiacanga could serve as
234 autonomous centers to deliver migrants to less prevalent neighborhoods, helping them to
235 achieve high and sustainable rates in the future, and providing a more uniform establishment
236 into Ilha do Governador. This effect, however, can only be verified after a continued long-
237 term monitoring of field populations, whose data may also indicate the necessity to apply
238 topic release boosts at those neighborhoods with persistent low rates. These considerations are
239 part of challenging large-scale release interventions, which are still incipient here in Brazil
240 and in other parts of the world (Schmidt et al. 2017; Ryan et al. 2019; Tantowijoyo et al.
241 2020). As a result of accumulating data from current trials, we shall better understand the
242 factors underlying invasion dynamics and optimize future strategies.
243 Altogether, our results ratify that *w*Mel field release is adaptable to large-scale, using
244 coordinated efforts to impact densely populated areas. With continuous improvement of
245 rearing and release technology, it could be amplified to cover city-wide territories in short
246 time. As preliminary disease impact studies suggest (Durovni et al. 2020; Pinto et al. 2021),
247 one could foresee a significant reduction in the incidence of dengue and other arboviral
248 diseases in Rio de Janeiro and nearby Niteroi, fulfilling the main goal of current trials and
249 cementing *Wolbachia* as an efficient and sustainable solution vector control in Brazil.
250

251 # Methods

252 **Mosquito husbandry**

253 To generate *w*MelRio, a precursor Australian line harboring the *w*Mel strain of *Wolbachia*
254 (Walker et al. 2011) was backcrossed for eight generations to a natural *Ae. aegypti* population
255 from Rio de Janeiro (Dutra et al. 2015). To achieve high genetic background homogenization,
256 additional crosses followed by *knockdown resistance* (*kdr*) screening were performed, and
257 individuals whose *kdr* profiling resembled that of the natural population were positively
258 selected (Garcia et al. 2019). To prevent drift and selection of new variants in our facilities,
259 and keep *w*MelRio in resonance with the natural background, our colony was refreshed every
260 five generations with 10% wild-caught males.

261 *w*MelRio eggs were hatched in degasified water with 0.08% Tetramin® (Tetra GmbH,
262 Herrenteich, Germany). After 5 h incubation at room temperature, hatching rates were
263 calculated and first instar (L1) larvae were transferred to mass-rearing trays containing 20-30k
264 individuals each. Larval development (L1 to L4) was promoted at 28 °C, in dechlorinated
265 water supplemented with liquid diet (3.7% fish meal, 2.6% liver powder and 1.1% brewer's
266 yeast). On the sixth day, with pupae formation reaching levels up to 50-70%, immatures were
267 collected and sent to either colony renewal or mass-release pipelines (see details for the latter
268 in 'Adult releases').

269 For colony renewal, immatures were split in groups of approximately 2,000
270 individuals and placed inside BugDorm® cages (MegaView Science Co Ltd, Taiwan). Adult
271 emergence and husbandry occurred at 25 °C, with 10% sucrose solution *ad libitum*. Females
272 were fed human blood (from donation centers; more details in 'ethical regulations') every 2-3
273 days, through Hemotek® artificial feeders (Hemotek Ltd, UK). Here, biosafety and ethical
274 guidelines were followed to prevent arboviral contamination of our colony and mass-release
275 batches, with all blood samples negatively scored for DENV, ZIKV, CHIKV, MAYV and

276 YFV by multiplex qPCR (Dutra et al. 2016; Pereira et al. 2018). For egg-collection,
277 dampened filter papers (i.e. half-immersed in water) were placed inside the cages for 2-3
278 days, before being removed and gradually dried at room temperature. Egg strips (a.k.a.
279 ovistrips) were stored at room temperature until further use, either for colony maintenance or
280 field release. Egg strips stored for more than 40 days were discarded due to a decay in overall
281 quality (Farnesi et al. 2019).
282
283 **Adult releases**
284 For the mass-release of *Wolbachia*-harboring *Ae. aegypti*, batches of approximately 150 late-
285 stage immatures were transferred to release tubes: custom-made acrylic pipes closed at both
286 ends with a fine mesh, allowing both liquid and air flow during the final developmental
287 stages. Following adult emergence, release tubes were counted, quality assessed and
288 designated to 'backpack' or 'vehicle' deployment.
289 For 'vehicle' deployment, release tubes were stacked into mini vans at dawn before
290 departing to trips covering a fraction of the release sites in Ilha do Governador. Each van
291 followed a strict routine, leaving the mass-production facility at scheduled times, and with the
292 driver and the release agent fully aware of the map, traffic and possible turnarounds. When
293 the van hit the approximate location of the sites, the agent would extend his/her arms outside
294 the window and gently remove the mesh to free the adults kept inside the tube. Once
295 completed, the van would proceed to the following site to repeat the procedure.
296 For 'backpack' deployment, release tubes were stacked inside backpacks before
297 departing to areas with restricted access, either because of irregular housing and narrow
298 alleys, or because of drug-related episodes of violence. In these areas, deployment was carried
299 out on foot by public health agents, working in partnership with both the WMP staff and
300 community leaders. As usual, before starting a trip, agents were given maps and routes to

301 cover the release sites, and asked to report their activity and any obstacle that might arise by
302 the end of the day.
303 The number and spatial distribution of release sites (Figure S1, Supplementary Table
304 S1) was strategically defined so as promote an efficient spread of *w*Mel-harboring individuals
305 into each neighborhood. Release sites were geotagged and integrated to ©OpenStreetMap
306 source data with ArcGIS version 10.4 (Esri, Redlands, CA, USA), allowing the planning of
307 daily routes and a better control and management over the whole release intervention.
308 Schedules (Supplementary Table S1) varied according to the area, RJ1 or RJ2, and
309 deployment method, 'vehicle' or 'backpack', being revisited after each round based on the
310 status of *Wolbachia* frequency in the field. Additional rounds were applied in order boost the
311 frequency levels and promote an efficient invasion.
312
313 **Ethical regulations**
314 Regulatory approval for the field release of *Wolbachia*-harboring *Ae. aegypti* was obtained
315 from the National Research Ethics Committee (CONEP, CAAE 02524513.0.1001.0008),
316 following a common agreement of governmental agencies (IBAMA, Ministry of
317 Environment; ANVISA, Ministry of Health; and MAPA, Ministry of Agriculture, Livestock
318 and Supply) and the former sanction of the special temporary registry (RET,
319 25351.392108/2013-96). Community acceptance was evaluated by social engagement
320 activities and a fill out questionnaire, with all neighborhoods recording > 70% household
321 support. Written informed consents were acquired from those hosting BG-sentinel traps, who
322 were offered financial aids to cover electricity costs.
323 Additional regulatory approval (CONEP, CAAE 59175616.2.0000.0008) was required
324 to feed the adult female mosquitoes with human blood, a necessary step for the maintenance
325 of *w*MelRio colony and mass production of eggs. We only used blood which would have been

326 discarded by not attending quality assurance policies (e.g. blood bags with insufficient
327 volume) of donation centers: Hospital Pedro Ernesto (Universidade Estadual do Rio de
328 Janeiro) and Hospital Antonio Pedro (Universidade Federal Fluminense). All blood samples
329 complied with Brazilian Government guidelines for routine screening, having no information
330 on donor's identity, sex, age and any clinical condition, as well as testing negative for
331 Hepatitis B, Hepatitis C, Chagas disease, syphilis, HIV and HTLV.
332

333 **Field population monitoring and *Wolbachia* diagnosis**

334 BG-Sentinel traps (Biogents AG, Regensburg, Germany) were spread across all
335 neighborhoods of Ilha do Governador (RJ) to monitor the *Wolbachia* frequency in the field
336 (Supplementary Figure S2, Supplementary Table S2). Monitoring sites covered an area of
337 approximately 250 m^2 each, and were selected among suitable households who formally
338 accepted hosting a trap. For an optimal control over the monitoring area and map creation,
339 sites were geotagged and overlayed with ©OpenStreetMap source data using ArcGIS version
340 10.4 (Esri, Redlands, CA, USA). Overtime, reallocation of sites was often necessary and
341 occurred when households quit hosting the trap, or in cases of equipment misuse or failure.
342 Staff agents checked each working trap weekly, bringing the catch bags (perforated envelopes
343 positioned inside the BG-Sentinels to trap insects) to our facilities for species identification
344 and *Wolbachia* screening.

345 *Ae. aegypti* samples were individually screened for *Wolbachia* by qPCR or LAMP. In
346 short, whole-bodies were homogenized in Squash Buffer (10 mM Tris-Cl, 1 mM EDTA, 25
347 mM NaCl, pH 8.2) supplemented with Proteinase K (250 ug/ml). DNA extraction was carried
348 out by incubating the homogenates at 56 °C for 5 min, followed by 98 °C for 15 min to stop
349 the proteinase activity. qPCR reactions were performed with LightCycler® 480 Probes
350 Master (Roche), using specific primers and probes to amplify *Wolbachia pipientis* WD0513

351 and *Ae. aegypti rps17* genes (Supplementary Table S3). Temperature cycling conditions were
352 set on a LightCycler® 480 Instrument II (Roche), using the following parameters: 95 ºC for
353 10 min (initial denaturation), and 40 cycles of 95 ºC for 15 s and 60 ºC for 30 s (single
354 acquisition). LAMP (*Loop-Mediated Isothermal Amplification*) reactions were performed
355 with WarmStart® Colorimetric LAMP 2X Master Mix (DNA & RNA) (New England
356 Biolabs) and an alternative set of primers (Supplementary Table S3), as described elsewhere
357 (Gonçalves et al. 2019). Isothermal amplification was carried out at 65 ºC for 30 min on a T-
358 100 Thermocycler (Bio-Rad), according with manufacturer conditions. Both qPCR and
359 LAMP reactions were performed in 96-well plates. Specimens with and without *Wolbachia*
360 were used as positive and negative controls, respectively.
361
362 ***kdr* genotyping**
363 Adult *Ae. aegypti* were genotyped by qPCR to detect single nucleotide polymorphisms
364 (SNPs) at the 1016 (Val^{+} or Ile^{kdr}) and 1534 (Phe^{+} or Cys^{kdr}) positions of the voltage gated
365 sodium channel gene (Na_V), as previously reported (Macoris et al. 2018; Hayd et al. 2020).
366 Amplification reaction was performed with LightCycler 480 Probes Master mix (Roche),
367 10ng of individual genomic DNA, and a set of primers and probes to detect *kdr* alleles
368 (Supplementary Table S3) customized by Thermo Fisher Inc. under ID codes: AHS1DL6
369 ($Val^{+}1016Ile^{kdr}$) and AHUADFA ($Phe^{+}1534^{Cys}$). Thermal cycling was carried out on a Light
370 Cycler 480 Instrument II (Roche), set to the following conditions: 95 ºC for 10 min (initial
371 denaturation), and *N* cycles of 95 ºC for 15 s and 60 ºC for 30 s (single acquisition). *N* was set
372 to 30, for amplifying $Val^{+}1016Ile^{kdr}$, or to 40, for $Phe^{+}1534Cys^{kdr}$. For each collection date, 30
373 samples were individually genotyped. Rockefeller colony specimens (kindly provided by Dr.
374 Ademir de Jesus Martins Júnior, IOC, Fiocruz), harboring susceptible (Na_vS) or resistant
375 variants (Na_vR1 and Na_vR2), were used as positive controls.

Statistical Analyses

All statistical analyzes were performed in Graphpad Prism 8 (Graphpad Software, Inc). *Wolbachia* frequency time-series were smoothed using a moving average of 7-neighbors, 2nd order polynomials. Spearman correlation *r* coefficient was used to compare invasion trends between great intervention areas, RJ1 and RJ2, as well as the deployment strategies applied within each, 'vehicle' or 'backpack'. For all statistical inferences, α was set to 0.05.

Data Availability Statement

All relevant data generated or analyzed during this study are included in this manuscript (and its Supplementary Information file).

Ethics Statement

This study and all the underlying work regarding the rearing and field-release of *Wolbachia*-harboring *Ae. aegypti* was approved by the National Research Ethics Committee (CONEP, CAAE 02524513.0.1001.0008), following a common agreement of governmental agencies (IBAMA, Ministry of Environment; ANVISA, Ministry of Health; and MAPA, Ministry of Agriculture, Livestock and Supply) and the former sanction of the special temporary registry (RET, 25351.392108/2013-96).

Author contributions

J.S.M.G., S.P., F.B.S.D., J.P., G.C. and L.A.M. conceived the study. J.S.M.G., S.P., F.B.S.D., J.P., G.C., S.K., J.M., B.R.G., P.A.R., C.P.S., K.L.A., S.L.O. and L.A.M. performed the investigation, data curation and analysis. L.A.M. managed the project supervision, validation

and funding. J.S.M.G., S.P. and L.A.M. drafted the manuscript, and all authors reviewed and approved its final version.

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Conflict of Interest

The authors have declared that no conflict of interests exists.

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625 ## Figure Legends

626 **Figure 1. Map of Ilha do Governador intervention areas and neighborhoods.** Satellite
627 view of Ilha do Governador area, the largest island of Guanabara bay, in northern Rio de
628 Janeiro (RJ). With an estimated total population of 211,018 in 40 km^2, Ilha do Governador is
629 divided into the following neighborhoods: Bancários, Cacuia, Cocotá, Freguesia, Monero,
630 Pitangueiras, Praia da Bandeira, Ribeira, Tauá, Zumbi, Cidade Universitária, Galeão, Jardim
631 Carioca, Jardim Guanabara, and Portuguesa. For *Wolbachia* release intervention,
632 neighborhoods were grouped into two great areas, RJ1 (green) and RJ2 (yellow). Note that
633 Cidade Universitária is actually located in an adjacent island, Ilha do Fundão, which is under
634 the same public administration zone of Ilha do Governador and was therefore included as part
635 of the RJ2 area. Also depicted is Tubiacanga (red), a small neighborhood which was targeted
636 in a pioneer release trial.
637

638 **Figure 2. *Wolbachia*'s introgression into Ilha do Governador.** Adult *w*Mel-harboring *Ae.*
639 *aegypti* were mass-released in RJ1 and RJ2 areas, covering the entire territory of Ilha do
640 Governador (Rio de Janeiro). Release intervention was carried out in three rounds (grey
641 shading). Invasion profiles are depicted separately for (A) RJ1 and (B) RJ2, plus an aggregate
642 of both for (C) an overall representation. Following the left Y-axis, *Wolbachia* prevalence

643 indexes (%) are color coded and plotted as dots plus 2nd degree, 7-neighbors, moving averages
644 (dashed lines). Following the right Y-axis, sample sizes are plotted as histograms (orange
645 bars). Time (weeks), since the beginning of adult releases (Week 1, August 2017) until recent
646 days (Week 131, March 2020), is represented in the X-axis. Ticks are scaled for 20-week
647 bins.
648
649 **Figure 3. *Wolbachia*'s invasion profiles in individual neighborhoods.** Adult *w*Mel-
650 harboring *Ae. aegypti* were released (grey shading) across all neighborhoods of Ilha do
651 Governador. Individual invasion profiles are depicted, with 'RJ1' (green) or 'RJ2' (purple)
652 coding for the intervention area, and 'V' or 'B' for vehicle or backpack-assisted releases,
653 respectively. *Wolbachia* prevalence indexes (%) are color coded and represented by dots plus
654 2nd degree, 7-neighbors, moving averages (dashed lines), following the left Y-axis. Sample
655 sizes are plotted as histograms (orange), following the right Y-axis. Prevalence indexes from
656 small-sized samples (N<5) are marked in red. The X-axis represents time (weeks), since the
657 beginning of adult releases (Week1, August 2017) until recent days of field monitoring (Week
658 131, March 2020), with ticks scaled accordingly to represent 20-week bins. Post-release
659 *Wolbachia* prevalence in Tubiacanga (blue), a previous intervention site, is shown as a
660 standard for long-term field establishment.
661
662 **Figure 4. Comparison of invasion profiles between intervention areas and deployment**
663 **strategies.** *w*Mel frequencies for different intervention areas, or deployment strategies, were
664 represented individually and overlayed, and compared by Spearman's correlation analyses. A)
665 RJ1 vs RJ2; B) RJ1.V vs RJ1.B; C) RJ2.V vs RJ2.B. The degree of association between
666 frequency datasets is indicated by the *r* coefficient, at the top right of the correlation graphs.
667

668 **Figure 5. Genetic monitoring of insecticide resistance in intervention areas.**

669 Knockdown resistance (*kdr*) allelic variants were monitored in field caught mosquitoes

670 (*Wolbachia*$^{+}$) over the release and post-release interventions in RJ1 and RJ2. Data represent

671 the proportion of alleles linked to susceptibility, S, or resistance to insecticides, R1 and R2, in

672 each sample (n=30). Mosquitoes from Tubicanga, home to a previous successful trial, were

673 also included for comparison. S (*Na$_v$S*): 1016Val^{+}; R1 (*Na$_v$R1*): 1016Val^{+}, 1534Cys^{kdr}; R2

674 (*Na$_v$R2*): 1016Ile^{kdr}, 1534Cys^{kdr}.

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Figure 1

Tubiacanga
Freguesia
Bancários
Moneró
Tauá
RJ 1
Portuguesa
Cocotá
Jardim Carioca
Galeão
RJ 2
Praia da Bandeira
Jardim Guanabara
Pitangueiras
Cacuia
Zumbi
Ribeira
Brazil
Atlantic Ocean
Rio de Janeiro
Niteroi
Cidade Universitária
0 1 2 km
Sources: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, the GIS User Community

Figure 2

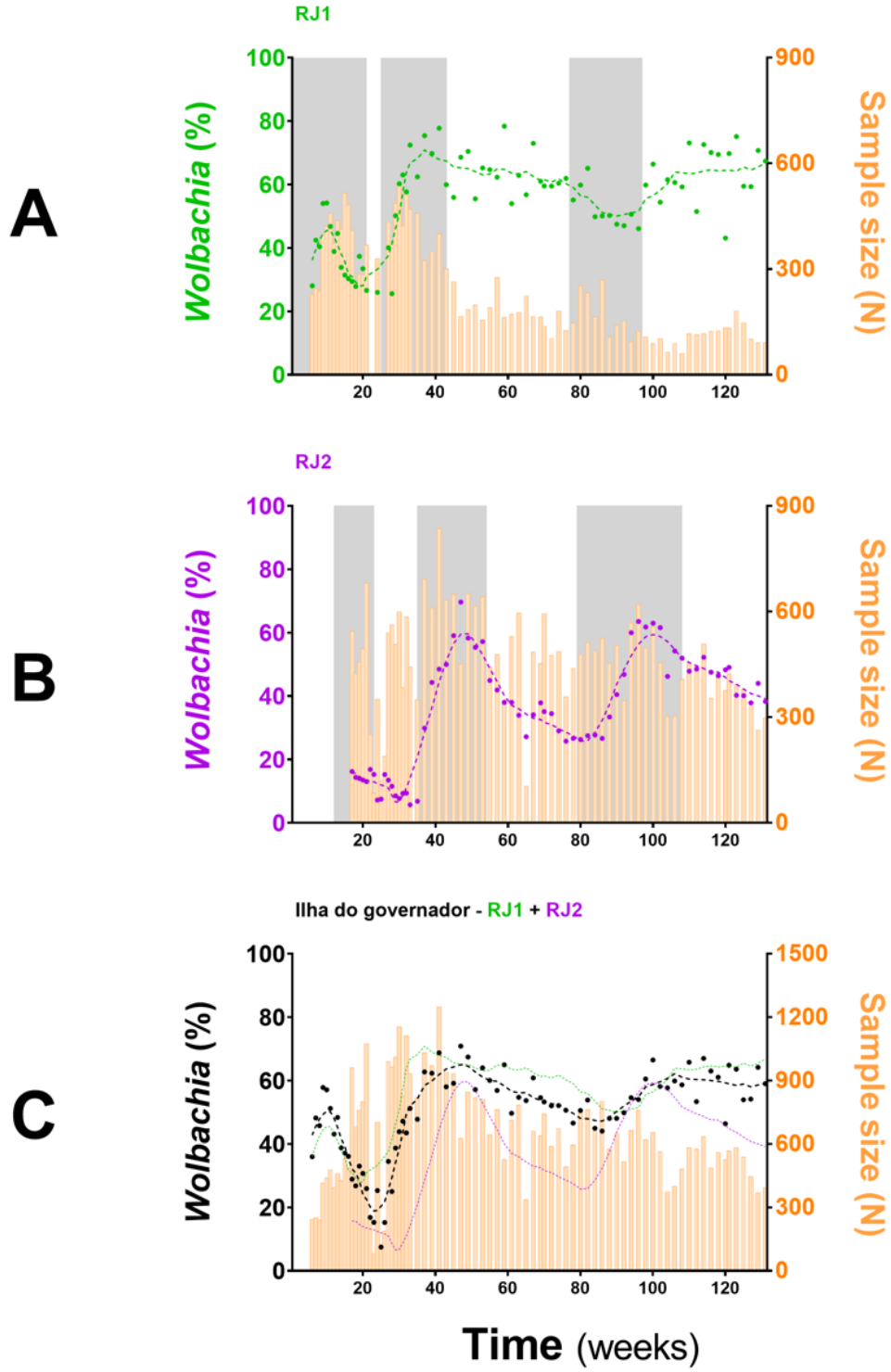

A
RJ1
Wolbachia (%)
Sample size (N)
B
RJ2
Wolbachia (%)
Sample size (N)
C
Ilha do governador - RJ1 + RJ2
Wolbachia (%)
Sample size (N)
Time (weeks)

Figure 3

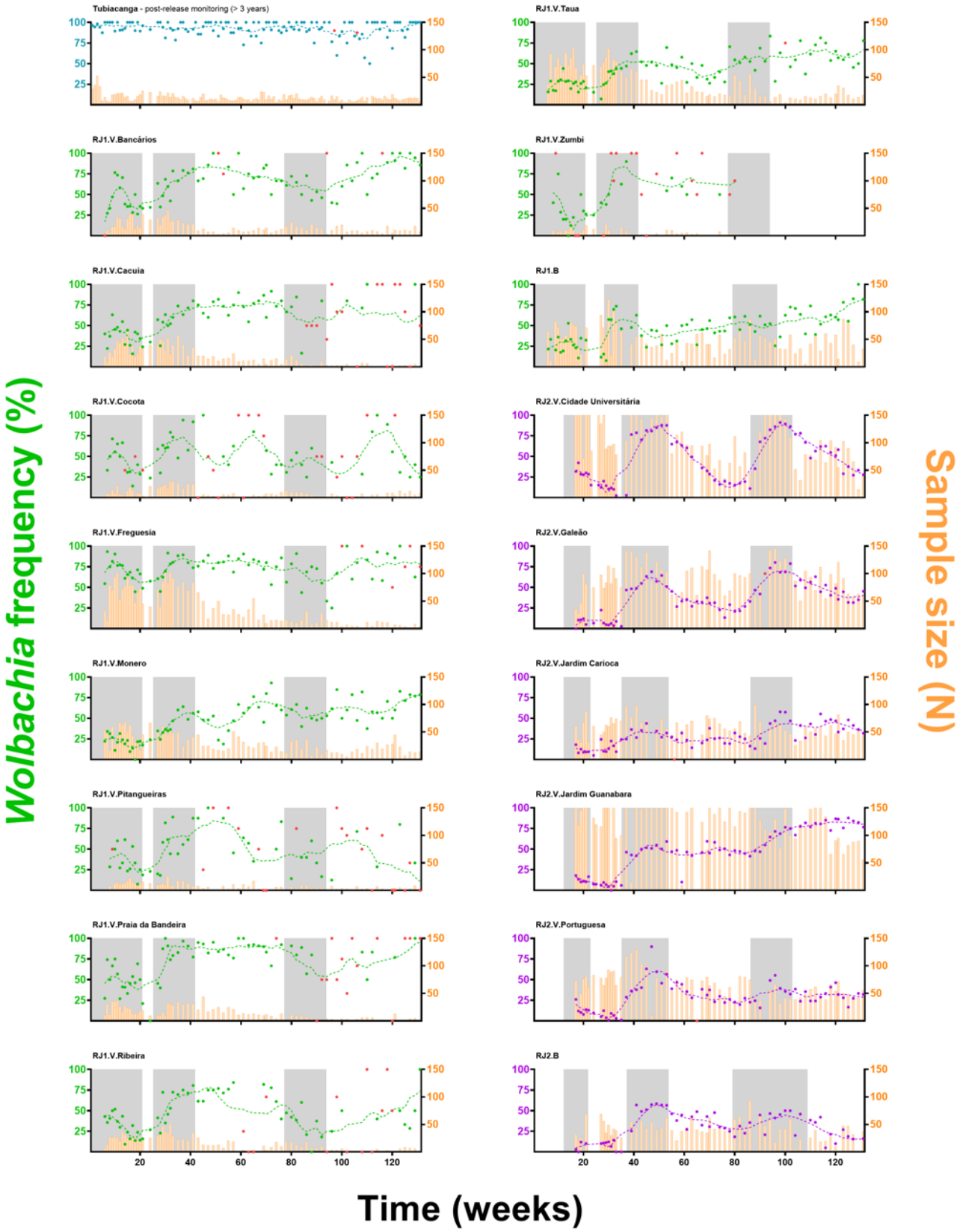
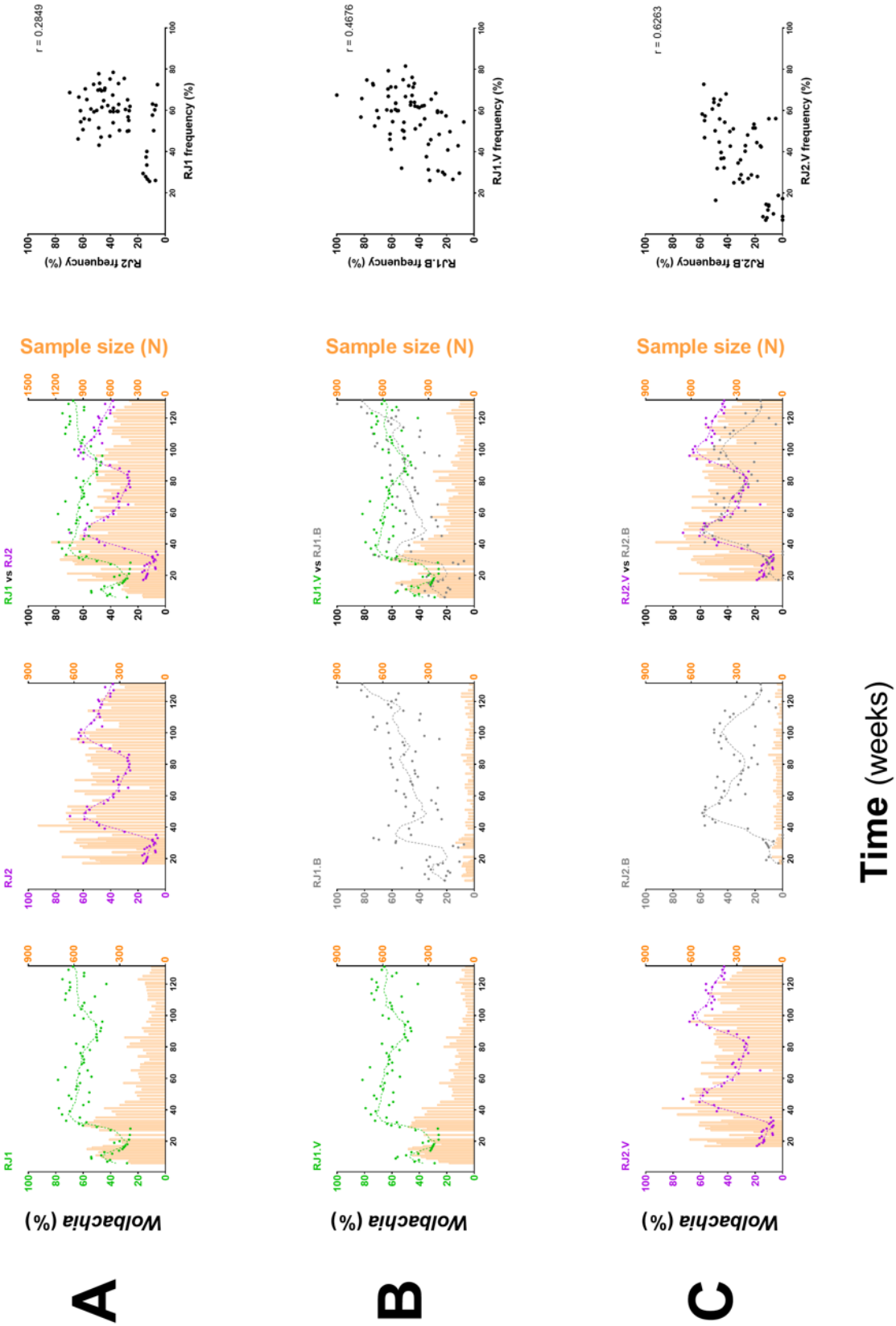
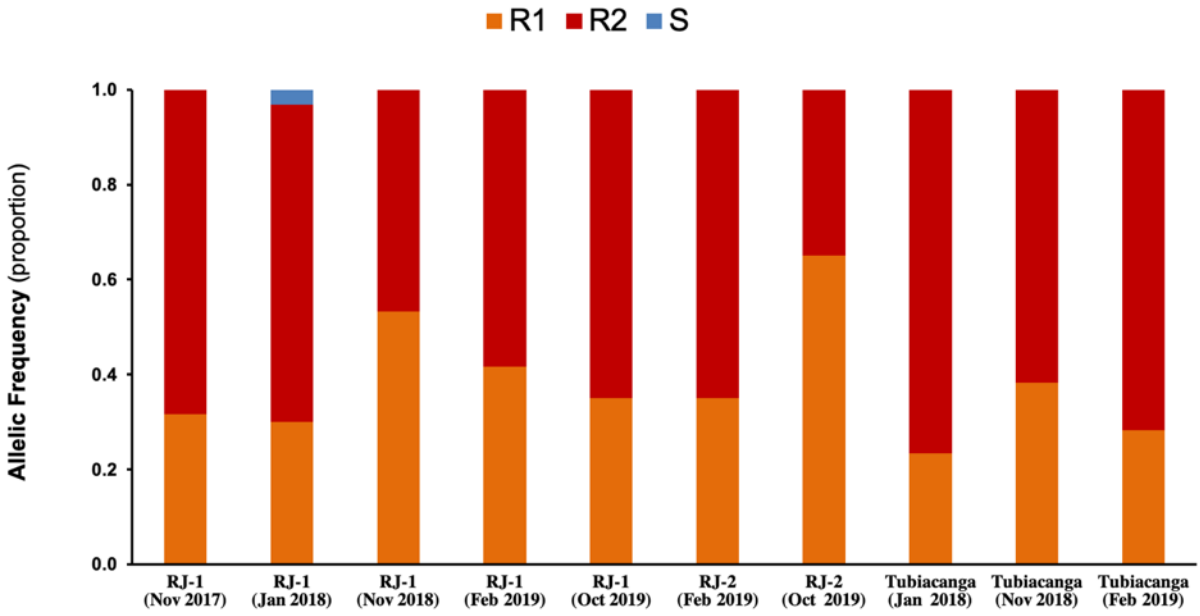

Tubiacanga - post-release monitoring (> 3 years)
RJ1.V.Taua
RJ1.V.Bancários
RJ1.V.Zumbi
RJ1.V.Cacuia
RJ1.B
RJ1.V.Cocota
RJ2.V.Cidade Universitária
RJ1.V.Freguesia
RJ2.V.Galeão
RJ1.V.Monero
RJ2.V.Jardim Carioca
RJ1.V.Pitangueiras
RJ2.V.Jardim Guanabara
RJ1.V.Praia da Bandeira
RJ2.V.Portuguesa
RJ1.V.Ribeira
RJ2.B
Wolbachia frequency (%)
Sample size (N)
Time (weeks)

Figure 4


A
B
C
Wolbachia (%)
Sample size (N)
RJ1
RJ2
RJ1 vs RJ2
RJ1.V
RJ1.B
RJ1.V vs RJ1.B
RJ2.V
RJ2.B
RJ2.V vs RJ2.B
Time (weeks)
r = 0.2849
RJ2 frequency (%)
RJ1 frequency (%)
r = 0.4676
RJ1.B frequency (%)
RJ1.V frequency (%)
r = 0.6263
RJ2.B frequency (%)
RJ2.V frequency (%)

718 **Figure 5**

719



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