Research Article

Received: 3 January 2023 Revised: 31 March 2023 Accepted article published: 6 April 2023 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.7495

A bacterium against the tiger: further evidence of the potential of noninundative releases of males with manipulated *Wolbachia* infection in reducing fertility of *Aedes albopictus* field populations in Italy

Beniamino Caputo,[a] Riccardo Moretti,[b] Chiara Virgillito,[a] Mattia Manica,[c] Elena Lampazzi,[b] Giulia Lombardi,[b,d] Paola Serini,[a] Verena Pichler,[a] Nigel W. Beebe,[e] Alessandra della Torre[a*] and Maurizio Calvitti[b*]

Abstract

BACKGROUND: Incompatible insect technique (IIT) is a population suppression approach based on the release of males with manipulated *Wolbachia* infection inducing egg inviability in wild females. We here present results of multiple field releases of incompatible AR*w*P males carried out in 2019 in a 2.7-ha green area within urban Rome (Italy) to assess the effect on *Aedes albopictus* egg viability. Data are compared with results obtained in 2018, when the approach was tested for the first time in Europe.

RESULTS: An average of 4674 AR*w*P males were released weekly for 7 weeks, resulting in a mean AR*w*P:wild male ratio of 1.1:1 (*versus* 0.7:1 in 2018). Egg-viability dynamics in ovitraps significantly varied between treated and control sites, with an estimated overall reduction of 35% (*versus* 15% in 2018). The estimated proportion of females classified as mated with AR*w*P males was 41.8% and the viability rate of eggs laid by these females (9.5%) was on average significantly lower than that of females only mated with wild males (87.8%); however, high variability in fertility was observed. Values of AR*w*P male competitiveness were 0.36 and 0.73 based on the overall viability rate of eggs in ovitraps and on female fertility, respectively; thus, well above the conventional 0.2 threshold for an effective suppressive impact in the field.

CONCLUSIONS: Results further support the potential of IIT as a tool to contribute to *Ae. albopictus* control in the urban context, stressing the need for larger field trials to evaluate the cost-efficacy of the approach in temperate regions.

Supporting information may be found in the online version of this article.

Keywords: mosquito; *Aedes albopictus*; incompatible insect technique; *Wolbachia*; open field trials; Italy; Europe

1 INTRODUCTION

Goals, approaches and investments for mosquito control are very different in temperate regions than in tropical ones where mosquito-borne diseases are endemic and cause over 700 000 deaths and hundreds of millions of human cases each year.[1,2] In Europe, since malaria eradication in the second half of the 20th

* *Correspondence to: A della Torre, Department of Public Health and Infectious Diseases, University of Rome La Sapienza, Rome, Italy. E-mail: alessandra.dellatorre@uniroma1.it; or M Calvitti, Biotechnology and Agroindustry Division, ENEA (Italian National Agency for New Technologies, Energy and Sustainable Economic Development), Casaccia Research Center, Rome, Italy. E-mail: maurizio.calvitti@enea.it*

Beniamino Caputo, Riccardo Moretti joint first Authors.

a *Department of Public Health and Infectious Diseases, University of Rome La Sapienza, Rome, Italy*

b *Biotechnology and Agroindustry Division, ENEA (Italian National Agency for New Technologies, Energy and Sustainable Economic Development), Casaccia Research Center, Rome, Italy*

c *Center for Health Emergencies, Fondazione Bruno Kessler, Trento, Italy*

d *School of Biosciences and Veterinary Medicine, University of Camerino, Camerino, Italy*

e *School of Biological Sciences, University of Queensland, & CSIRO, Brisbane, Australia*

Century, the most concerning pathogen transmitted by mosquitoes has been the West Nile Virus.[3,4] However, globalization and climate change are extending the public health threats related to these insects as a consequence of the introduction and spreading of invasive species competent for exotic diseases.[5,6] In spite of this, mosquitoes are still perceived almost exclusively as a source of (sometimes relevant) nuisance by people in Europe.[7] This creates different prospects in the fight against mosquitoes. On the one hand, the development of novel strategies for reduction of mosquito nuisance and risk of disease transmission in temperate regions can benefit of the more intensive research focusing on main tropical vectors species. On the other, the adaptation of novel strategies to vector species in temperate regions must consider biological differences among species and eco-climatic and sociological differences between the regions, as well as differences in the goals of the proposed approaches and in potential investments, and be consistent with local regulations. Moreover, the assessment of the efficacy of control interventions is more challenging in temperate regions, where the assessment of the reduction of clinical cases cannot be taken as the final endpoint of the control, as done in endemic regions such as Singapore.[8]

In Europe, the main vector of exotic arboviruses is *Aedes albopictus*, an invasive Asian species which in the last three decades has colonized all Mediterranean countries and is expanding northwards to central European regions,[9,10] as well as north America.[11] In the last two decades, the species has been responsible of autochthonous transmission of chikungunya (CHIKV) and dengue (DENV) viruses in Europe and it has caused two CHIKV epidemics in 2007 and 2017 in Italy,[12] and DENV outbreaks in 2020 in Italy[13] and in 2022 in France.[6] Moreover, owing to its diurnal and aggressive biting behaviour, the species has changed the citizen's habits by affecting their outdoor activities and causing economic damage in the touristic and recreational activity sectors.[7,14] Larval control is the recommended measure for prevention and reduction of *Ae. albopictus* nuisance and risk of arbovirus transmission in Europe.[15,16] In Italy, for instance, most municipalities invest a (more or less relevant) budget each year for calendar-based larvicide treatments of street catch basins and to educate citizens to reduce or treat larval sources in private areas. However, in areas and periods of high nuisance, aerial spraying of pyrethroid adulticides also is frequently carried out for fast mosquito abatement, with high economic and environmental costs, as shown by raising levels of resistance to these compounds.[17–19]

Under this scenario, novel eco-friendly approaches originally developed to fight tropical vectors of other insect pests are being studied and tested in order to adapt them to the specific needs of fighting *Ae. albopictus* in temperate nonendemic countries and complement existing mosquito control methods.[20] Among the most promising and advanced ones are population suppression approaches based on the release of males capable of reducing fertility of wild females either because they are sterilized by irradiation (sterile insect technique, SIT)[21] or as a result of manipulation of their *Wolbachia* endosymbiont (incompatible insect technique, IIT).[21] The latter approach is based on a phenomenon of egg infertility occurring in crosses between males infected by an incompatible *Wolbachia* strain, absent in the wild population, and wild-type (WT) females. Given that *Wolbachia* is inherited through the female germline, once an opportune infection has been established, the obtained colony can be maintained to produce and release incompatible males, generation by generation, without any further manipulation.

Effectiveness and economic sustainability of these strategies should be analysed carefully before planning large investments for their scaling up in temperate regions, taking into account production costs and expected benefits for the target area in terms of reduction of nuisance, health-related costs and preserved environmental safety. IIT field tests conducted in recent years have provided encouraging information regarding the potential of the strategy.[22] Nevertheless, an increase in open-field IIT experiments over environmentally diversified areas is needed in order to get reliable data to evaluate the cost-effectiveness of this approach.

In 2018, we conducted, for the first time in Europe, multiple field releases of males from an *Ae. albopictus* line (i.e. the AR*w*P line, deprived of the natural *Wolbachia* infection and transinfected with a *Culex pipiens Wolbachia* strain inducing a bidirectional pattern of incompatibility with WT *Ae. albopictus*),[23] with the goal of assessing the effect on egg viability and wild female fertility over time.[24] Despite the small scale of the effort, encouraging results were obtained. We here present results from an additional series of releases of AR*w*P males carried out in 2019 in the same study area in Rome (Italy), with the following goals: (i) to assess the replicability of the results obtained in 2018 and compare results; (ii) to estimate mating competitiveness of AR*w*P males under field conditions; and (iii) to deepen knowledge regarding safety issues related to the possible release of small numbers of contaminant AR*w*P females.

2 MATERIAL AND METHODS

The methods described in the following paragraphs largely replicate those described in Caputo *et al.*[23] Nonetheless, we describe them here in detail to help the readers to easily follow the results.

2.1 ARwP male production: sexing and assessment of the insemination of contaminant females

ARwP males were produced at ENEA Casaccia laboratories. Rearing was carried at a larval density of 2 larvae mL^{-1} in deionized water, at 28 °C, 80% relative humidity (RH) and a 14 h:10 h, light:dark photoperiod. Larvae were fed for 4 days with increasing doses (0.2, 0.4, 0.6 and 0.8 mg larva^{-1} day^{-1}) of a liquid diet consisting of 50% tuna meal, 36% bovine liver powder, 14% brewer's yeast and 0.2% w/v vitamin mix (IAEA-BY diet).[25] Twenty-four hour-old pupae were sexed mechanically by passing them through a 1400-μm metal sieve over 3 min at 34 °C.[26] After swarming, adults were kept at 15 °C for 24 h and then knocked-down by chilling to 10 °C allowing for manual removal of residual females. Selected males were kept at 25 ± 1 °C and 80% RH in cubic plastic cages (30 cm; Bugdorm1, MegaView Science Co., Ltd, Taichung, Taiwan) and fed with 10% sugar solution until release. All contaminated females were dissected to check the possible occurrence of insemination during the first 24 h before they were separated from males.

2.2 Study sites and ARwP *Ae. albopictus* male releases

Open release field trials involving AR*w*P *Ae. albopictus* males were authorized for research purposes by the Italian Ministry of Health on 22 May 2018. The releases were carried out in central Rome (Italy) in highly *Ae. albopictus* infested sites within the premises of Sapienza University (2.7 ha; WGS84-UTM33 coordinates = 294 181, 4 643 934; Fig. 1), after authorization by Sapienza Technical Office. Two other green areas with ecological conditions similar to those at the treated site were selected as control

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

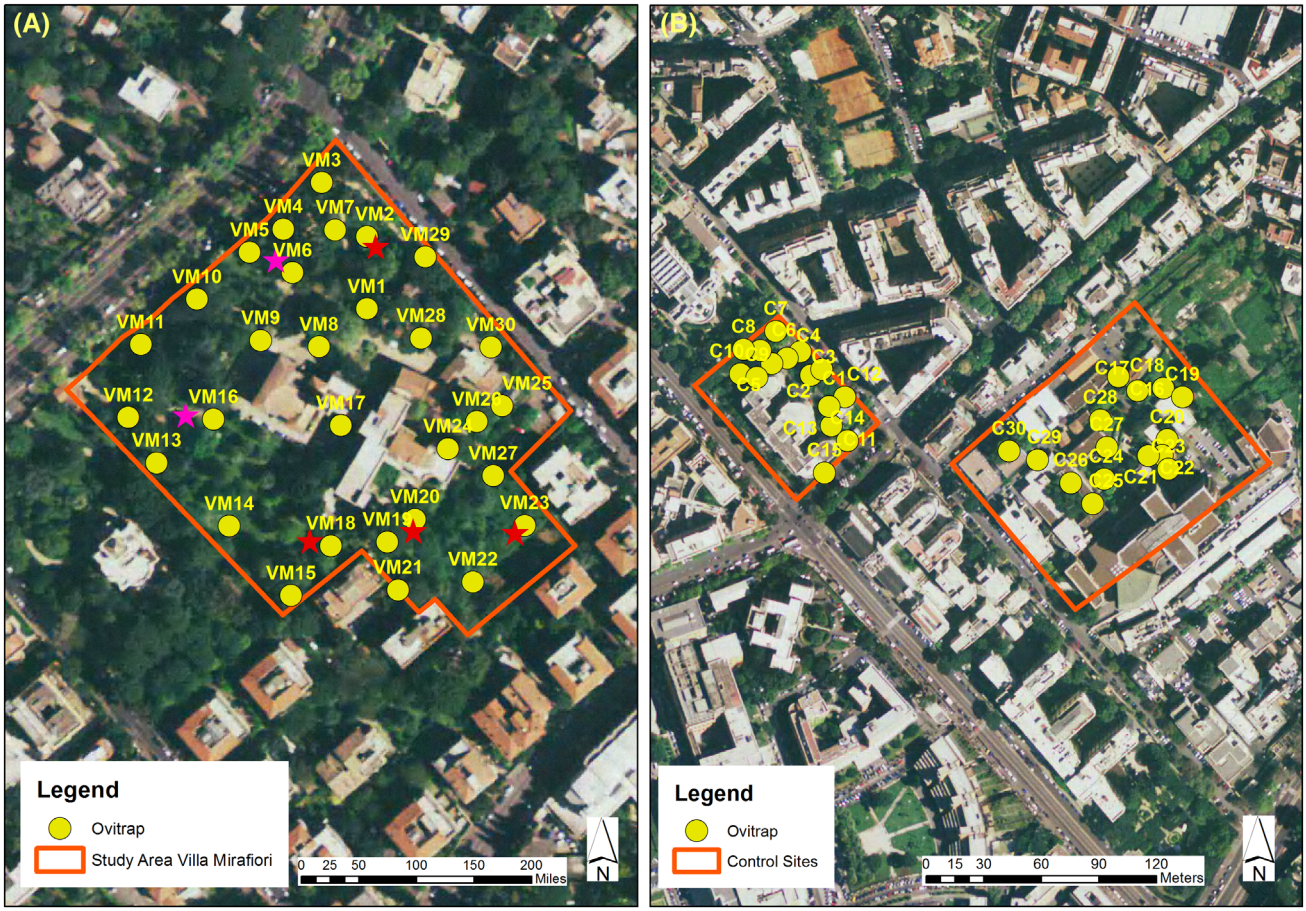

Figure 1. Study sites in the premises of Sapienza University (Rome, Italy). (A) Treated site (Department of Philosophy). (B) Control sites (Institute of Anatomy and Department of Engineering). Yellow dots, position of ovitraps in treated (n = 30) and control (n = 30) sites; red stars, release spots of ARwP males, where also adult male and female collections were carried out; pink stars, release spots of ARwP males, where collections were not carried out (from Caputo *et al*. 2020).

sites (coordinates = 294 257, 4 642 526). More details on treated and control sites are available in Caputo *et al*.[24]

ARwP males (1–2 day old) were transferred by car from ENEA Casaccia laboratories to the treated site in <1 h inside 30 × 30 × 30 cm plastic cages (each containing ~850 individuals) and released in the same six spots selected for 2018 experiments. Releases were carried out weekly in the late afternoon from 21 June to 30 July.

2.3 Egg collection by ovitraps and viability assessment

Aedes albopictus eggs were collected twice a week during the morning hours by 30 ovitraps in the treated site and 30 in control sites from 30 May to 9 August. Ovitraps consisted of black plastic vases with an overflow hole 3 cm below the upper border, filled with 500 mL tap water. A wooden paddle with one rough side was placed in each ovitrap. During each monitoring, water was refilled, wooden paddles collected and replaced with new ones. Wooden paddles collected from ovitraps were transferred to SAPIENZA laboratories in individual plastic bags and handled as described in Caputo *et al*.[24] At the end of the hatching process, the eggs were examined under a microscope and classified as either viable (i.e. either hatched or embryonated) or sterile (i.e. not presenting any recognizable embryo). Viability rates were calculated as the ratio between the number of eggs classified as hatched/embryonated out of the total eggs examined.

2.4 Field collection of *Ae. albopictus* females and assessment of fertility in single females

From 18 June to 9 August, immediately before every ARwP male releases, *Ae. albopictus* females were collected by manual aspirations (~5min/spot) in the proximity of the six release spots in the treated site and in three spots in each of the two control sites, and transferred to 30 cm × 30 cm × 30 cm plastic cages. The cages were transported to either Sapienza or ENEA laboratories, where females were blood-fed the following day. On the Day (D) 3 after the blood meal, individual females were transferred to oviposition cups lined with filter paper and allowed to lay eggs for the following 4 days. Eggs were treated following the same protocol described above for eggs collected by ovitraps and eventually classified as either viable or sterile. Adult females were frozen and kept at −20 °C. Spermathecal capsules were examined under a microscope to test whether females showing 100% inviable eggs had been inseminated.

2.5 Field collections of *Ae. albopictus* males and identification of ARwP males

Aedes albopictus males were collected with a mosquito-net from 08 00 to 10 00 h on the same days of ovitrap monitoring by a single operator acting for about 15 min in each of the six release spots of the treated site and were interrupted 6 days after the last male release. Collected specimens were labelled and stored in

single tubes with 70% ethanol to be later processed to detect the possible presence of *w*Pip Wolbachia (see below) and identify released *versus* field males.

2.6 Field collections of *Ae. albopictus* eggs and assessment of *w*Pip infection in treated site

A subset of 20 eggs per release spot were collected in the treated site 1 week after the last release. Eggs were hatched and larvae reared to adulthood. Emerged females were labelled and stored in single tubes with 70% ethanol to be later processed to detect the possible presence of *w*Pip *Wolbachia* (see below), which would indicate that females had escaped from the sexing procedure, mated with AR*w*P males and laid viable eggs.

2.7 Identification of wPip *Wolbachia* in field collected specimens

DNA was extracted by homogenizing the abdomens of male or female mosquitoes in 100 μL STE with 0.4 mg mL^{-1} proteinase K.[27] AR*w*P individuals were identified by polymerase chain reaction (PCR) using primers *w*PF (5′-CGACGTTAGTGGTG-CAACATTTA-3′) and *w*PR (5′-AATAACGAGCACCAGCAAAGAGT-3′)[28] which amplify a DNA sequence specific to the *w*Pip *Wolbachia* strain *wsp*-gene.[29] The PCR conditions used were: 94 °C for 5 min followed by 32 cycles of 94 C for 30 s, 54 C for 30 s, 72 C for 40 s and a single final step at 72 C for 10 min. Amplified fragments were electrophoresed on 1.5% agarose gels, stained with ethidium bromide (1 μg mL^{-1}) and visualized under UV light.

2.8 Statistical analysis

Statistical analyses were carried out using R software v4.1.3.[30] Regression models were applied in a Bayesian framework using JAGS v4–12.[31] For all regression models, model assumptions were checked by graphical inspection of model residuals. To estimate parameters of all models, three chains were used running 50 000 iterations with a burn-in of 40 000 and a thinning rate of 10.

2.8.1 *Assessment of the viability rate of* Ae. albopictus *eggs over time*

The temporal dynamic of the viability rate – here estimated as the ratio between the number of viable (i.e. hatched and embryonated) eggs and the total number of collected eggs – was investigated using a binomial generalized additive mixed model (GAMM-1) with logit link. It should be highlighted that viability rate was calculated based on eggs classified as hatched/embryonated on the total of hatched, embryonated and sterile eggs.[24] Model covariates were site (qualitative:treated/control) and day of the year (quantitative). The day of the year was included as a smoothing function (O'Sullivan spline with five internal knots) to model the nonlinear temporal effect of the viability rate in the two sites. The interaction between day of the year and site also was considered to allow different temporal patterns of viability rate between treated and control sites. Day of the year was standardized (subtracted its mean value and divided by its standard deviation, SD) to improve the numerical stability of the model and help with interpretation of the results. The mixed-effects model approach with the random-effect ovitrap was considered to take into account that observations were collected repeatedly from each ovitrap during the experiment.

Missing values and ovitrap data with zero eggs collection were discarded. Diffuse normal priors [Norm (0,1000)] were used for the smoothers and covariate parameters Given that high viability was expected in the control site (here used as reference), we used informative prior [Norm (3,1)] for the intercept of the model. Chauchy priors were used for the variance terms of both smoothers and random effect [$\sigma^2{}_1 \sim$ |Norm(0,25) / Norm(0,1)|]. For further details see Caputo *et al*. (2020).[24]

2.8.2 *Assessment of fertility rates of single ovidepositing* Ae. albopictus *females*

The fertility rate of single females collected in the field was investigated by analysing the viability of individual eggs batches using a binomial generalized linear model (GLM-1) with logit link. Because a female mosquito in the treated site may or may not have mated with AR*w*P males, mosquito mating was modelled as a Bernoulli trial. If the female was modelled as AR*w*P-mated then the viability rate of its eggs would differ from that of a non-AR*w*P-mated female, which is assumed to correspond to the eggs viability rate of single females collected in the control sites. Beta priors were used for both viability rates [AR*w*P-mated females: Beta(a = 1, b = 20); non-AR*w*P-mated females: Beta(a = 1, b = 1)] and the mosquito AR*w*P mating [Beta(a = 1, b = 1)]. This is a simplification that does not explicitly model multiple mating. For further details see Caputo *et al*. 2020.[24]

2.8.3 *Comparison of temporal dynamics of reduction in* Ae. albopictus *egg viability in 2018* versus *2019 field experiments*

The reduction in egg viability rate (*R*) is defined as the subtraction between observed egg viability rate in the control and in the treated sites. The temporal dynamics of *R* were investigated using a generalized additive model (GAM-1). Model covariates were year of the experiment (2018 and 2019) and days of the year. The days of the year were included as a smoothing function (O'Sullivan spline with four internal knots) to model the nonlinear temporal effect of the viability rate in the two years. The interaction term between days of the year and year of the experiment also was considered to allow different temporal patterns of *R* between the two years. *R* was modelled assuming a normal distribution with parameters mean μ_i and variance σ^2. GAM-1 equations are as follows:

$$R_i \sim Norm\left(\mu_i, \sigma^2\right)$$
$$E(R_i) = \mu_i; Var(R_i) = \sigma^2$$
$$\mu_i = \alpha + \beta Year_{i,k} + f_k(Day_i) + \varepsilon_i$$

where R_i is the reduction observed at collection i ($i = 1, \ldots, 16$), α and β are the regression parameters, $f_k()$ is the smoothing function where the index k indicates a different smoother per year (2018, 2019) and ε_i is the error term assumed to follow a normal distribution [Norm (0, σ^2)]. Diffuse normal priors [Norm (0,1000)] were used for the smoother parameters and for regression parameters, Chauchy priors were used for the variance terms of both smoothers, whereas uniform distribution (0,1) was used for the standard deviation of the normal distribution.

2.8.4 *Male competitiveness index*

The competitiveness index *C* of a sterile male population corresponds to the odds of a wild female being mated with an incompatible male compared to being mated with a wild male when exposed to both type of males in equal numbers. A *C*-value of 1 indicates that sterile and wild males are equally competitive, whereas a *C*-value of

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

0.5 indicates that females are two times more likely to be mated with wild males.[32] C is defined by Fried's index,[33] as:

$$C = \frac{\left(\frac{H_a - E}{E - Hs}\right)}{R_t}$$

where H_a is the observed viability rate of eggs in the control site, E the observed viability rate of eggs in the treated site, H_s is the residual fertility of males and R_t is the ratio of incompatible wild males in time window t. In the present work, t was arbitrarily chosen within 7 days after the first release of ARwP males and 6 days after the last release, when the biases on ovidepositing females due to mating with wild males before and after the first and last release is expected to be lower. H_s often is neglected when the residual fertility of males is <1% as in the case of the crosses between ARwP males and WT *Ae. albopictus* females.[28] It is worth noting that the exact value of C could only be measured in a confined environment where migration is not allowed and wild females are virgin at start. Here, conscious of the possible biases discussed below, we estimated two values of C under open field conditions, based on different observed fertility rates in time window t: C_1, calculated based on the total viability rate of eggs in ovitraps (defined as sum of number of viable, i.e. hatched or unhatched but embryonated, eggs divided by the total number of collected eggs) in control and treated sites; C_2, calculated based on the measurement of the fertility rate of single ovipositing females collected close to the release spots. Embryonated eggs were conservatively assigned to the category of viable eggs even if it is known that, when bidirectional incompatibility occurs, a proportion of the embryonated eggs can be represented by defective embryos unable to hatch.[34] Finally, a sensitivity analysis was carried out using the mean viability rate of eggs collected in the ovitraps in the treated site before the first release. For each value of C, we computed the confidence intervals with the method of percentile bootstrap[35] (based on 1000 bootstrap replicates at the 95% level).

3 RESULTS

A total of 32 721 ARwP males (corresponding to an average of 4674 males per week) were released during the 7-week experiment (Table 1). Inspection of adult mosquitoes before release led to the removal of 102 contaminant females, corresponding to an average of 0.3% females on the total of released specimens. None of these females was inseminated (Table S4).

3.1 Ratio of ARwP to wild *Ae. albopictus* males

A total of 423 *Ae. albopictus* males was collected in the six release spots within the treated site (Table 1). Twenty males per collection date (or less if not available) were processed by PCR to identify presence of *w*Pip *Wolbachia* sequences. ARwP male frequencies of 80% and 53.3% were observed at D3 and D6 after the first release, respectively. ARwP male frequency ranged between 40% and 60% during the following weeks, reached 90% at D2 from last release and was still 40% at D6 after the last release. The mean frequency of ARwP males, collected between D3 after the first release and D4 after the last release, was 54.1% (95% CI 23.49–84.61), corresponding to a mean ARwP:wild male ratio of 1.12:1.

3.2 Viability rates of eggs collected by ovitraps

A total of 17 317 *Ae. albopictus* eggs (6036 in treated site, 11 281 in control sites) were collected in 718 ovitrap/collections (304 in treated site, 414 in control site) (Table S1). A total of 572 ovitrap/collections with no eggs or missing values was found (341 in the treated site, 261 in control sites). The mean viability rate observed in the period encompassing 7 days after the first release and 6 days after the last one was 71.2% and 99.3% in treated and control sites, respectively, with an overall reduction of fertility in the treated site of 29%. According to GAMM-1, viability rate before the first release (calculated for 2604 and 5189 eggs collected in treated and control sites, respectively) was >96% with no statistical difference between sites, as shown by the overlapping credible intervals in Fig. S1. Overall, the mean percentage of viable eggs estimated by GAMM-1 from D1 after the first release to 7 days after the last release was 68.2%

Table 1. Releases and recaptures of ARwP *Aedes albopictus* males in summer 2019 in a green area within urban Rome (Italy)

Date of release	Released ARwP males	Days since last release	Collected *Ae. albopictus* males	*Ae. albopictus* males tested by PCR	ARwP male frequency (n)
21 June	4800	-	-	-	-
24 June	-	3	68	20	80% (16)
27 June	-	6	15	15	53.3% (8)
28 June	4800	7	-		-
1 July	-	3	34	20	45% (9)
4 July	3850	6	12	12	41,7% (5)
8 July	1420	2	30	20	60% (12)
11 July	4435	3	39	20	55% (11)
15 July	-	4	24	20	50% (10)
18 July	-	7	18	18	38.9% (7)
19 July	5461	8	-		-
22 July	-	3	58	20	50% (10)
25 July	4395	6	22	20	40% (8)
29 July	-	4	17	17	58.8% (10)
30 July	3560	5	-		-
1 August	-	2	39	20	90% (18)
5 August	-	6	47	20	40% (8)

Abbreviation: ARwP male frequency, number of ARwP males/total number of collected males tested by PCR.

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

(average absolute deviation = 30.2%) and 98.6 (average absolute deviation = 1.72%) for eggs collected in treated and control sites, respectively. Egg viability rate in the treated site showed a strong temporal pattern (Figs 2, S1) and was on average significantly lower than in control sites (Table 2).

Figure 2 shows the estimated temporal dynamics of egg viability rates based on GAMM-1. Overall, the estimated viability rate was always >96% in control sites, whereas in the treated site it decreased immediately after the first ARwP-male release, dropping to ~60% concurrently with the fifth release, when the highest difference between viability rates in treated and control sites was recorded (35% at D22 from first release; i.e. D195 in Fig. 2). Viability rate in the treated site was <80% during the following three releases and increased 10 days after the last release.

3.3 Fertility rates of single wild *Ae. albopictus* females after ArwP male releases

The fertility rate of single ovipositing females collected between D3 from the first ArwP male release and D6 after the last release was assessed by examining 4736 eggs laid by 122 females collected in the treated site and 2349 eggs laid by 60 females from control sites (Table S2). The observed mean fertility rate between 7 days after the first release and 6 days after the last one was 52% and 95% in treated and control sites respectively, with on overall reduction of fertility in the treated site of 43%. Moreover, 23% of females collected in the release spots laid 100% infertile eggs; all were confirmed to be inseminated by the presence of sperm in the spermathecae. Results of GLM-1 (Table 3) showed a bimodal pattern of viability in eggs laid by females collected in the treated site, as opposed to a high viability rate in eggs laid by females collected in control sites (Fig. 3). Based on GLM-1 estimates, the proportion of females classified as mated with ARwP males in the treated site was 41.8% (95% CI 33.18–50.80%) (black dots in Fig. 3). The viability rate of eggs laid by these females (9.48%; 95% CI 8.06–9.99%) was on average significantly lower than that of females classified as not-mated with ARwP males in both treated and control sites (87.8%; 95% CI 86.60–88.82%) (vertical bars in Fig. 3).

3.4 Comparison of reduction in *Ae. albopictus* egg viability in 2018 *versus* 2019 field experiments

Based on GAM-1, the reduction in egg viability after ARwP male releases was on average significantly higher in 2019 (35%; CI 27%–41%) than in 2018 (15%; CI 7%–22%) (Table S3; Fig. 4). A steeper temporal pattern is observed in 2019, when the reduction in egg viability reached its peak after the seventh release, as opposed to 2018 when the peak reduction was observed after the third release. In both years, the effect of a reduction in eggs viability was observable within 10 days after the first release (see confidence intervals excluding zero in Fig. 4) and maintained by subsequent releases.

3.5 Male competitiveness index

The overall *C*-value computed over the period encompassing 7 days after the first ARwP male release and 7 days after the last release was estimated to be 0.36 (95% CI 0.33–0.45) based on

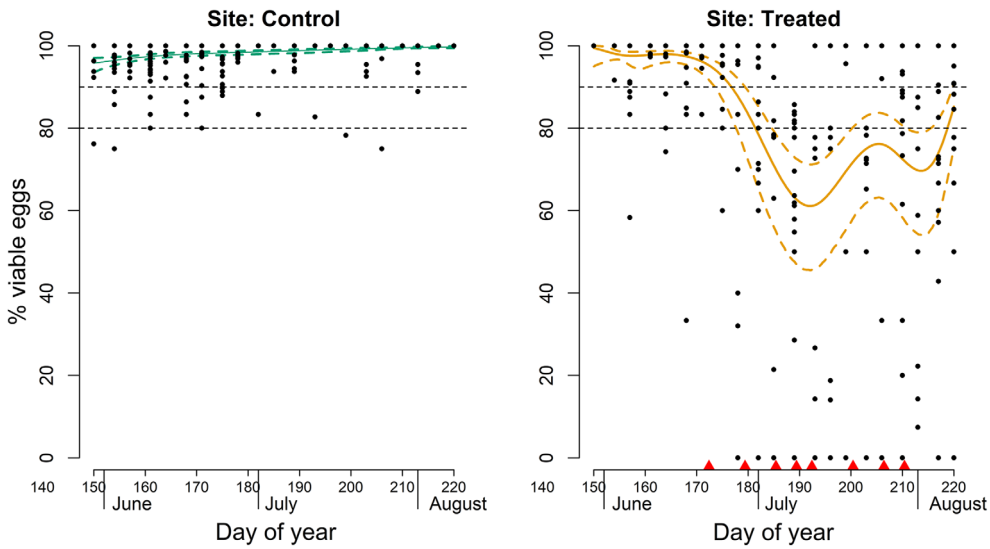

Figure 2. Temporal dynamics of the percentage of viable *Aedes albopictus* eggs collected in treated and control sites in Rome (Italy) in 2019, as estimated by GAMM-1. Solid lines, estimated mean percentages of egg viability; dashed lines, 95% credible intervals; black dots, observed percentage of viable eggs/ovitrap; red triangles, ARwP male release dates.

Table 2. Model parameters and viability rate of *Aedes albopictus* eggs collected in treated and control sites in Rome (Italy) in 2019, as estimated by GAMM-1

Site	Mean	SE	95% CI	Viability rate: mean (95% CI)
Control	4.223	0.164	3.922/4.566	98.6
Treated	−1.364	0.343	−2.228/−0.901	68.2

Note: Estimated mean values and 95% credible intervals (CI) of model parameters at a logit scale and of viability rate for control and treated site.

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Table 3. GLM-1 estimates of fertility rate of *Aedes albopictus* females classified as mated and unmated with ARwP males and of the expected probability of mating with ARwP males in the treated site

Description	Mean (%)	SE	95%CI (%)
Mated	9.48	0.522	8.06/9.99
Unmated	87.76	0.562	86.60/88.82
Probability of mating with ARwP males	41.78	4.580	33.18/50.80

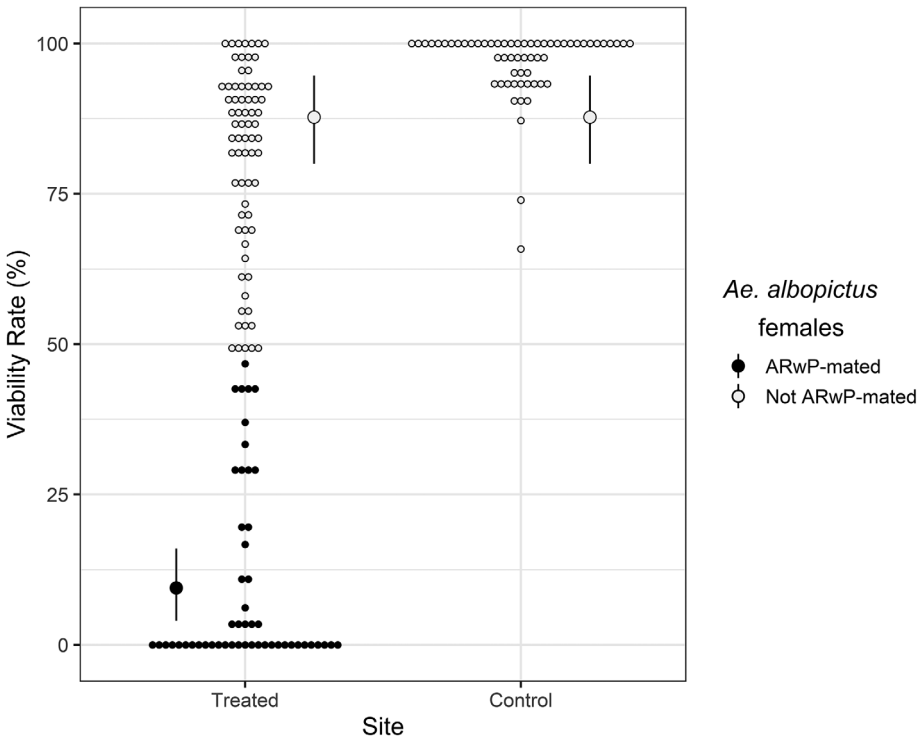

Figure 3. Egg viability in single ovipositing *Aedes albopictus* females collected in treated and control sites, as estimated by GLM-1. Observed viability rates are shown as small dots coloured depending on model classification as AR*w*P-mated (black) or not mated (white), each dot represents the egg viability of a single ovipositing females. The estimated mean viability rate of single ovipositing females is shown as large points coloured following the same colour scheme for AR*w*P-mated (black) or not mated (white); vertical solid lines represent the 95% prediction intervals.

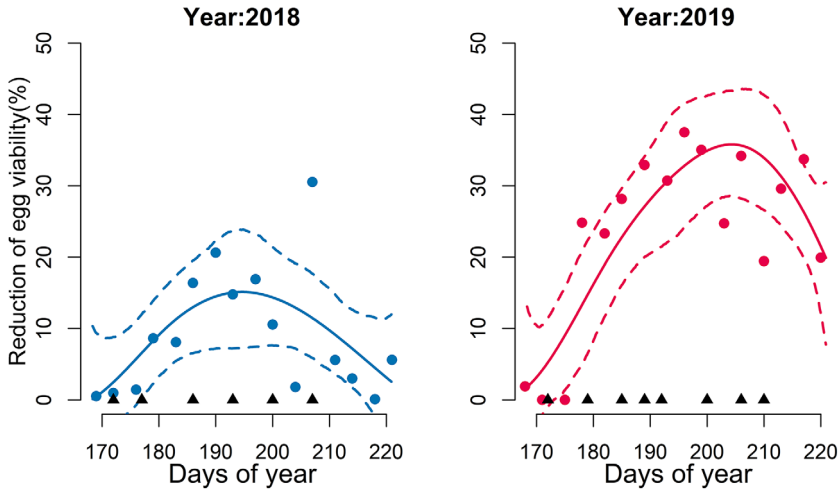

Figure 4. Temporal dynamics of the reduction in viability rate of *Aedes albopictus* eggs after AR*w*P male releases in 2018 and 2019, as estimated by GAM-1. solid lines, estimated percentage of reduction in the treated site compared to the control site; dashed lines, 95% credible intervals; points, observed percentage reduction in the treated site compared to the control site; triangles, dates of AR*w*P male releases.

total viability of eggs collected in the ovitraps. The sensitivity analysis revealed a comparable result for *C* when the observed mean fertility of eggs collected in each ovitraps in the treated site before the first release was used as control in Fried's eqn (0.39, 95% CI 0.35–0.48). When *C* was calculated based on the total fertility rate of single ovipositing females, its value was estimated to be 0.73 (95% CI 0.64–0.98). Adding the embryonated eggs to the category of the viable eggs did not significantly affect the results (Fig. S2), as these correspond to <2% of total eggs collected.

3.6 Assessment of *w*Pip infection in eggs from the treated site

*w*Pip *Wolbachia* was not detected in any of the 49 females obtained from the eggs collected at the release spots 1 week after the end of the releases (Table S5).

4 DISCUSSION

An IIT trial targeting *Ae. albopictus* was conducted in 2019 in a small green area in Rome, replicating an experimental scheme implemented in 2018 in the same area and during the same season (late June–early August).[24] A higher reduction of viability of eggs collected by ovitraps was estimated in 2019 (35% *versus* 15% in 2018). Consistently, a higher mean ratio between AR*w*P and wild males was observed in 2019 (1.1:1 *versus* 0.7:1 in 2018). Notably, the estimate of the incompatible:sterile wild male ratio was very straightforward thanks to an effective male marking system stable throughout the whole male life: the *w*Pip *Wolbachia*-specific PCR-assay.[28,36] This represents an asset of IIT in comparison to SIT trials in the field, as the latter requires marking of irradiated male releases with fluorescent dusts, which progressively lose detectability over time and can be passed to other males during fights.[37]

In order to evaluate the potential efficacy and sustainability of AR*w*P male release as a potential control strategy, we estimated the per-male efficiency associated to the incompatible strain, by taking into account not only the incompatible:wild male ratio, but also male survival.[32] A male competitiveness value (C_1) of 0.36 was estimated based on the overall viability rate of eggs in ovitraps in 2019. This value is higher than the *C*-value estimated for 2018 experiments (0.21) and lies in the upper side of the range of *C*-values observed in similar field studies conducted with irradiated *Ae. albopictus* males.[32,38,39] Both 2018 and 2019 *C*-values are above the 0.2 threshold, which is considered as the lower limit for an effective suppressive impact on the target population in the field.[32]

In addition to the above standard approach to assess of viability in eggs laid in ovitraps, we collected single females inside the release area in order to assess the effect of cytoplasmic incompatibility at the individual level rather than in the whole population. Interestingly, 23% of these females were completely sterile, as expected when wild females mate only with AR*w*P males.[40,41] The estimated probability of mating between wild females and AR*w*P males (41.8%; CI 33.2–50.7%) is consistent with the observed *C*-value of 0.73 (C_2) calculated based on the overall fertility rate of single ovidepositing females and the 1.1:1 ratio between AR*w*P and wild males assessed based on PCR-identification of *w*Pip *Wolbachia*-specific DNA sequences. The difference between the two *C*-values – C_1 calculated based on the egg viability in ovitraps (representing all the monitored area) and C_2 based on egg fertility of females collected close to the release spots (representing an area at higher density of incompatible males and further from the boundaries of the studied area) – highlights how this parameter is sensitive to the context.

The statistical model applied also estimates that the mean fertility of females mated at least once with AR*w*P males is 9.5%, as opposed to 87.5% in females mated with wild males. However, high variability in fertility values was observed among analyzed females. As already hypothesized in Caputo *et al.* (2020), this may be a result of multiple insemination, a phenomenon already documented in *Ae. albopictus*,[42,43] which needs to be better quantified owing to its potentially high impact on the effectiveness of IIT, as well as of any kind of SIT approach. In this respect, it is important to note that both 2018 and 2019 experiments were carried out in a small green area within a highly anthropized environment, and that under this experimental setting immigration of females already mated by wild males out of the study area is very likely. This might account for the higher overall observed mean reduction of fertility in single females (43%) that were captured closer to the release points, as opposed to the mean reduction in viable eggs from ovitraps (29%) scattered in the whole study area. It is likely that the proportion of females immigrated in the study site from neighbouring areas (and probably mated with wild males in advance) is higher among females laying eggs in ovitraps than in females collected within the release area (where they are more likely to have emerged and mated). We expect that increasing the size of the treated area would reduce the effects of these immigration events in the centre of the area and increase the proportion of females exclusively inseminated by AR*w*P males and, consequently, the mean proportion of inviable eggs.

Regarding the replacement risk issues,[44] it is relevant to stress that the implemented sexing protocol, although not automatized, did not allowed any AR*w*P females to be inseminated by the AR*w*P males before release. Together with the bidirectional incompatibility pattern occurring between AR*w*P and WT *Ae. albopictus*, this contributes to virtually eliminate any risk of AR*w*P spread in the field, even in the case of unintentional releases of females, especially when releases are not inundative.[41]

5 CONCLUSION

Altogether, results obtained in 2018 and 2019 provide evidence supporting incompatible male release as a promising method to complement conventional control approaches against *Ae. albopictus* in urban/peri-urban areas in temperate regions. The ecological characteristics of the study site and the relative low number of released males are likely to have led to an underestimation of the sterilizing capacity of AR*w*P males, owing to immigration of females inseminated out of the release site by wild males and to the dispersal of incompatible males. In fact, the high AR*w*P male competitiveness and the indications of their high longevity (the relative ratio between incompatible and wild males was still 40% 4 days after the last release) accounts for high AR*w*P male mating efficiency.

AR*w*P male frequency among collected males after the release and the estimated temporal trends suggest that the frequency of release was adequate to maintain a living AR*w*P male population in the field during the study period. However, the detectable but limited reduction in egg viability found in ovitraps suggests that the control strategy would benefit by increasing the number of released males and release points. It is worth further stressing that these results were obtained by noninundative releases of males

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

reared under standard laboratory conditions with no automatized mass rearing tools. This release scenario leaves large opportunities for increasing the capability for larger incompatible male releases that would lead to higher incompatible:wild male ratios and higher levels of sterility.[22,45] Implementing IIT in association with conventional treatment of major larval sites, for instance, would allow the incompatible:wild male ratio to increase by reducing the wild male population and targeting the females emerged from cryptic breeding sites. Notably, the bidirectional incompatibility pattern between AR*w*P and WT *Ae. albopictus* represents a relevant safety feature, limiting the unintended spread of AR*w*P females, because, in nonisolated bidirectional CI-based systems, the less frequent infection type tends to be rapidly eliminated.[41,46,47]

Larger scale field studies are needed to eventually assess the effectiveness and long-term sustainability of IIT in contributing to reduce *Ae. albopictus* abundance and, possibly, prevent the risk of exotic arbovirus transmission in urban areas in temperate regions. Also, it will be crucial to evaluate the cost-effectiveness of this approach – either as a stand-alone or as an integrated control strategy – in geographical regions where arboviruses are not endemic and the species almost exclusively represents a nuisance problem.

ACKNOWLEDGEMENTS

We are grateful to Sapienza University Ufficio Tecnico Settore V for authorizing the study and helping with the logistics, and to the Departments of Filosofia (Villa Mirafiori), Biologia e Biotecnologie Charles Darwin and of Scienze di Base Applicate per l'Ingegneria for hosting the experiments. This research was partially supported by EU funding within the MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (project no. PE00000007, INF-ACT, research node 2).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AdT, BC, MC, RM. Performed the experiments: BC, EL, GL, MB, NWB, PS, VP, RM, MC. Analyzed the data: BC, CV, MM. Contributed reagents/ materials/ analysis tools: AdT, BC, MC. Wrote the paper: AdT, BC, CV, MC, MM, RM. All co-authors revised the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 WHO (World Health Organization), Global Vector Control Response 2017–2030 [Internet]. Geneva (2017). Available from: https://www.ptonline.com/articles/how-to-get-better-mfi-results.
2 Tozan Y, Branch O and Rocklöv J, Vector-borne diseases in a changing climate and world, in *Climate Change and Global Public Health*. In Springer, Switzerland, pp. 253–271 (2021).
3 Brugman VA, Hern LM, Medlock JM, Fooks AR, Carpenter S and Johnson N, The Role of Culex pipiens L. (Diptera: Culicidae) in Virus Transmission in Europe (2018).
4 Zeller HG and Schuffenecker I, West Nile virus: an overview of its spread in Europe and the Mediterranean basin in contrast to its spread in the Americas. *Eur J Clin Microbiol Infect Dis* **23**:147–156 (2004).
5 Gossner CM, Ducheyne E and Schaffner F, Increased risk for autochthonous vector-borne infections transmitted by Aedes albopictus in continental Europe. *Euro Surveill* **23**:1800268 (2018) Available from: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2018.23.24.1800268.
6 Cochet A, Calba C, Jourdain F, Grard G, Durand GA, Guinard A *et al*., Autochthonous dengue in mainland France, 2022: geographical extension and incidence increase. *Euro Surveill* **27**:2200818 (2022).
7 Halasa YA, Shepard DS, Fonseca DM, Farajollahi A, Healy S, Gaugler R *et al*., Quantifying the impact of mosquitoes on quality of life and enjoyment of yard and porch activities in new Jersey. *PLoS One* **9**: e89221 (2014).
8 Soh S, Ho SH, Seah A, Ong J, Dickens BS, Tan KW *et al*., Economic impact of dengue in Singapore from 2010 to 2020 and the cost-effectiveness of Wolbachia interventions. *PLOS Glob Public Health* **1**:e0000024. Available from: (2021). https://doi.org/10.1371/journal.pgph.0000024.
9 Metelmann S, Caminade C, Jones AE, Medlock JM, Baylis M and Morse AP, The UK's suitability for Aedes albopictus in current and future climates. *J R Soc Interface* **16**:20180761 (2019) Available from: https://royalsocietypublishing.org/doi/abs/10.1098/rsif.2018.0761.
10 Kuhlisch C, Kampen H and Walther D, The Asian tiger mosquito Aedes albopictus (Diptera: Culicidae) in Central Germany: surveillance in its northernmost distribution area. *Acta Trop* **188**:78–85 (2018).
11 Hahn MB, Eisen RJ, Eisen L, Boegler KA, Moore CG, McAllister J *et al*., Reported distribution of *Aedes* (*Stegomyia*) *aegypti* and *Aedes* (*Stegomyia*) *albopictus* in the United States, 1995–2016 (Diptera: Culicidae). *J Med Entomol* **53**:1169–1175 (2016) Available from: https://academic.oup.com/jme/article-lookup/doi/10.1093/jme/tjw072.
12 Caputo B, Russo G, Manica M, Vairo F, Poletti P, Guzzetta G *et al*., A comparative analysis of the 2007 and 2017 italian chikungunya outbreaks and implication for public health response. *PLoS Neglected Trop Dis* **14**:1–12. Available from: (2020). https://doi.org/10.1371/journal.pntd.0008159.
13 Lazzarini L, Barzon L, Foglia F, Manfrin V, Pacenti M, Pavan G *et al*., First autochthonous dengue outbreak in Italy, august 2020. *Euro Surveill* **1**:8–11 (2020).
14 Darbro J, Halasa Y, Montgomery B, Muller M, Shepard D, Devine G *et al*., An economic analysis of the threats posed by the establishment of Aedes albopictus in Brisbane, Queensland. *Ecol Econ* **142**:203–213. Available from: (2017). https://doi.org/10.1016/j.ecolecon.2017.06.015.
15 Bellini R, Michaelakis A, Petrić D, Schaffner F, Alten B, Angelini P *et al*., Practical management plan for invasive mosquito species in Europe: I. Asian tiger mosquito (Aedes albopictus). *Travel Med Infect Dis* **35**: 101691 (2020).
16 ECDC, Guidelines for the surveillance of invasive mosquitoes in Europe (2012).
17 Pichler V, Bellini R, Veronesi R, Arnoldi D, Rizzoli A, Lia RP *et al*., First evidence of resistance to pyrethroid insecticides in Italian Aedes albopictus populations after 26 years since invasion. *Pest Manag Sci* **74**: 1319–1327 (2018).
18 Pichler V, Malandruccolo C, Paola S, Bellini R, Severini F, Toma L *et al*., Phenotypic and genotypic pyrethroid resistance of Aedes albopictus, with focus on the 2017 chikungunya outbreak in Italy. *Pest Manag Sci* **75**:2642–2651 (2019) Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/ps.5369.
19 Pichler V, Giammarioli C, Bellini R, Veronesi R, Arnoldi D, Rizzoli A *et al*., First evidence of pyrethroid resistance in Italian populations of West Nile virus vector Culex pipiens. *Med Vet Entomol* **36**:390–395 (2022).
20 Baldacchino F, Caputo B, Chandre F, Drago A, della Torre A, Montarsi F *et al*., Control methods against invasive *Aedes* mosquitoes in Europe: a review. *Pest Manag Sci* **71**:1471–1485. Available from (2015). http://doi.wiley.com/10.1002/ps.4044.
21 Achee NL, Grieco JP, Vatandoost H, Seixas G, Pinto J, Ching-Ng L *et al*., Alternative strategies for mosquito-borne arbovirus control. *PLoS Neglected Trop Dis* **13**:1–22 (2019).

22 Beebe NW, Pagendam D, Trewin BJ, Boomer A, Bradford M, Ford A *et al.*, Releasing incompatible males drives strong suppression across populations of wild and Wolbachia-carrying Aedes aegypti in Australia. *Proc Natl Acad Sci U S A* **118**:e2106828118 (2021).
23 Calvitti M, Moretti R, Lampazzi E, Bellini R and Dobson SL, Characterization of a new Aedes albopictus (Diptera: Culicidae)-Wolbachia pipientis (Rickettsiales: Rickettsiaceae) symbiotic association generated by artificial transfer of the wPip strain from Culex pipiens (Diptera: Culicidae). *J Med Entomol* **47**:179–187 (2010).
24 Caputo B, Moretti R, Manica M, Serini P, Lampazzi E, Bonanni M *et al.*, A bacterium against the tiger: preliminary evidence of fertility reduction after release of Aedes albopictus males with manipulated Wolbachia infection in an Italian urban area. *Pest Manag Sci* **76**:1324–1332 (2020).
25 Zheng ML, Zhang DJ, Damiens DD, Lees RS and Gilles JRL, Standard operating procedures for standardized mass rearing of the dengue and chikungunya vectors Aedes aegypti and Aedes albopictus (Diptera: Culicidae)-II-egg storage and hatching. *Parasites Vectors* **8**:1–7. Available from: (2015). https://doi.org/10.1186/s13071-015-0951-x.
26 Bellini R, Calvitti M, Medici A, Carrieri M, Celli G and Maini S, Use of the sterile insect technique against Aedes albopictus in Italy: first results of a pilot trial, in *Area-Wide Control of Insect Pests*, ed. by Vreysen MJB, Robinson AS and Hendrichs J. Springer Netherlands, Dordrecht, pp. 505–515 (2007).
27 Xi Z, Dean JL, Khoo C and Dobson SL, Generation of a novel Wolbachia infection in Aedes albopictus (Asian tiger mosquito) via embryonic microinjection. *Insect Biochem Mol Biol* **35**:903–910 (2005).
28 Calvitti M, Moretti R, Skidmore AR and Dobson SL, Wolbachia strain wPip yields a pattern of cytoplasmic incompatibility enhancing a Wolbachia-based suppression strategy against the disease vector Aedes albopictus. *Parasites Vectors* **5**:254 (2012) Available from: http://parasitesandvectors.biomedcentral.com/articles/10.1186/1756-3305-5-254.
29 Zhou W, Rousset F and O'Neil S, Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences. *Proc Biol Sci* **265**:509–515 (1998).
30 R Development Core Team, *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria (2022).
31 Plummer M, rjags: Bayesian Graphical Models using MCMC. R package version 4–12. [Internet] (2021). Available from: https://cran.r-project.org/package=rjags.
32 Bouyer J and Vreysen MJB, Yes, irradiated sterile male mosquitoes can Be sexually competitive! *Trends Parasitol* **36**:877–880 (2020).
33 Fried M, Determination of sterile-insect competitiveness. *J Econ Entomol* **64**:869–872. Available from: (1971). https://doi.org/10.1093/jee/64.4.869.
34 Bonneau M, Landmann F, Labbé P, Justy F, Weill M and Sicard M, The cellular phenotype of cytoplasmic incompatibility in Culex pipiens in the light of cidB diversity. *PLoS Pathog* **14**:e1007364 (2018).
35 Efron B and Tibshirani RJ, *An Introduction to the Bootstrap*. Chapman and Hall, New York (1993).
36 Bellini R, Albieri A, Balestrino F, Carrieri M, Porretta D, Urbanelli S *et al.*, Dispersal and survival of Aedes albopictus (Diptera: Culicidae) males in Italian urban areas and significance for sterile insect technique application. *J Med Entomol* **47**:1082–1091 (2010).
37 Rojas-Araya D, Alto BW, Cummings DAT and Burkett-Cadena ND, Differentiation of multiple fluorescent powders, powder transfer, and effect on mating in Aedes aegypti (Diptera: Culicidae). *Insects* **11**:727 (2020).
38 Bellini R, Carrieri M, Balestrino F, Puggioli A, Malfacini M and Bouyer J, Field competitiveness of Aedes albopictus (Diptera: Culicidae) irradiated males in pilot sterile insect technique trials in northern Italy. *J Med Entomol* **58**:807–813 (2021).
39 Velo E, Balestrino F, Kadriaj P, Carvalho DO, Dicko A, Bellini R *et al.*, A mark-release-recapture study to estimate field performance of imported radio-sterilized male Aedes albopictus in Albania. *Front Bioeng Biotechnol* **10**:833698 (2022).
40 Puggioli A, Calvitti M, Moretti R and Bellini R, wPip Wolbachia contribution to Aedes albopictus SIT performance: advantages under intensive rearing. *Acta Trop* **164**:473–481 (2016).
41 Moretti R, Marzo GA, Lampazzi E and Calvitti M, Cytoplasmic incompatibility management to support incompatible insect technique against Aedes albopictus. *Parasites Vectors* **11**:649 (2018).
42 Moretti R and Calvitti M, Male mating performance and cytoplasmic incompatibility in a wPip Wolbachia trans-infected line of Aedes albopictus (Stegomyia albopicta). *Med Vet Entomol* **27**:377–386 (2013).
43 Boyer S, Toty C, Jacquet M, Lempérière G and Fontenille D, Evidence of multiple inseminations in the field in Aedes albopictus. *PLoS One* **7**:1–5 (2012).
44 Yen P-S and Failloux A-B, A review: Wolbachia-based population replacement for mosquito control shares common points with genetically modified control approaches. *Pathogens* **9**:404 (2020).
45 Zheng X, Zhang D, Li Y, Yang C, Wu Y, Liang X *et al.*, Incompatible and sterile insect techniques combined eliminate mosquitoes. *Nature* **572**:56–61 (2019).
46 Dobson SL, Marsland EJ and Rattanadechakul W, Mutualistic Wolbachia infection in Aedes albopictus: accelerating cytoplasmic drive. *Genetics* **160**:1087–1094 (2002).
47 Dobson SL, Fox CW and Jiggins FM, The effect of Wolbachia-induced cytoplasmic incompatibility on host population size in natural and manipulated systems. *Proc Biol Sci* **269**:437–445 (2002).

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.