Combining two Genetic Sexing Strains allows sorting of non-transgenic males for *Aedes* genetic control

Authors

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Abstract

Chemical control of the mosquito vectors *Aedes albopictus* and *Aedes aegypti* is costly, unsustainable, and increasingly ineffective due to the spread of insecticide resistance. The Sterile Insect Technique is an autocidal control tactic that represents a valuable alternative but is limited by the slow, error-prone, and wasteful sex-separation stage.

Here, we present four genetic sexing strains (two for each *Aedes* species) based on fluorescence markers linked to the m and M sex loci, allowing the isolation of transgenic males. Furthermore, we show how combining these sexing strains allows the production of non-transgenic males. Scaling-up would allow the sorting of 100,000 neonate male larvae in under 1.5 hour with 0.01-0.1% female contamination. The resulting males present similar survival and flight ability to laboratory-reared wild-type males.

By facilitating the sorting of transgenic or non-transgenic males, these Genetic Sexing Strains should enable a major upscaling in control programmes against these major vectors.

Keywords

Vector control, M-locus, sex loci, *Aedes albopictus*, *Aedes aegypti*, COPAS, sex sorting, Sterile Insect Technique, Incompatible Insect Technique, mosquito control

Introduction

Aedes aegypti and *Aedes albopictus* are invasive mosquito species involved in the transmission of many pathogens including dengue (DENV), chikungunya (CHIKV), Zika (ZIKV) and yellow fever (YFV) viruses [1,2]. Driven by climate change and worldwide trade, both vectors are spreading rapidly and it is predicted that 49% of the world population will be at risk of *Aedes*-borne diseases by 2050 in the absence of effective control measures [3,4].

Suppression of mosquito populations using genetic control is one of the most effective, sustainable and environmentally friendly alternatives to insecticide use. It relies on repeated mass releases of male mosquitoes – either sterile (the Sterile Insect Technique, SIT [5]), *Wolbachia* infected (the Incompatible Insect Technique, IIT [6–8]), both [9,10], or carrying a lethal transgene (Release of Insects carrying a Dominant Lethal, RIDL [11].) For all these interventions, an efficient sex separation method is required. Currently, *Aedes* mosquitoes are sexed based on pupal size dimorphism using a Hoch's sorter, which can be either fully manual [12,13] or partly automated [10]. In both cases, this method suffers from female contamination rates between 0.8 and 1% and high daily and user-to-user variability [14]. Notably, a multi-step pupal and adult sorter was recently described which allowed 1.13 x 10^{-7}% female contamination [8]. However, all these methods sort at a late stage and recover less than half of the reared males [13], meaning that > 75% of total pupae are reared and fed in vain.

In *Anopheles* mosquitoes, transgenic genetic sexing strains (GSSs) allowing automated sex separation of young larvae have been described [15–17]. Fluorescent markers show male-specific expression, either by using male-specific regulatory sequences or by linking markers to the Y chromosome. One *Anopheles coluzzii* sexing strain is X-linked [18], rendering females more fluorescent than males. Sex separation is accomplished using a Complex Object Parametric Analyzer and Sorter (COPAS) device, which functions as a flow cytometer, sorting large particles according to their fluorescence. Additionally, as an alternative to the release of transgenic males, a crossing scheme generating non-transgenic male-only populations using COPAS was proposed [19]. This scheme requires a strain carrying a fluorescence marker on the Y chromosome and another carrying a fluorescence marker on the X chromosome. Crossing non-transgenic (X-/X-) females from the first strain to transgenic (X+/Y-

) males from the second results in progeny consisting of transgenic (X+/X-) females and non-transgenic (X-/Y-) males.

In *Aedes* mosquitoes, GSSs allowing sex-sorting of transgenic males exist. Two studies have proposed the use of a repressible female-specific flightless phenotype [20,21]. In this system, transgenic males and females are released together but adult females do not survive long as they are unable to fly. A method combining such sex-sorting and sterilization has been developed and adapted to *Ae. aegypti* for SIT [22,23]. However, this system requires perfect sex-sorting of the parental strains to be crossed, otherwise fertile transgenic mosquitoes (some of them homozygous for a *Cas9* transgene) could be released. Recently, we developed a GSS for *Ae. albopictus* carrying a fluorescence marker within a masculinizing transgenic cassette, allowing automated sorting of transgenic males [24].

Here, we examined if a crossing scheme similar to the one for *Anopheles* could be designed in *Aedes* mosquitoes despite the absence of heteromorphic sex chromosomes. In *Aedes*, sex is encoded by non-homologous sex loci located on the first pair of autosomes. The male locus is called “M”, while the common locus is called “m”; thus, females are m/m and males are m/M. These sex loci are about 1.18 Mbp long in *Ae. aegypti* [25], and are delimited by antagonistic factors that protect them from recombination [26,27]. They are embedded within a 63 Mbp region with strong male-female genetic differentiation, in which recombination remains suppressed [28]. Consequently, linking a fluorescence marker transgene to the *Aedes* sex loci or to the surrounding non-recombining region might allow automated sorting of either transgenic males directly from an M-linked strain, or of non-transgenic males after crossing hemizygous m-transgenic males with wild-type females **(Figure 1)**.

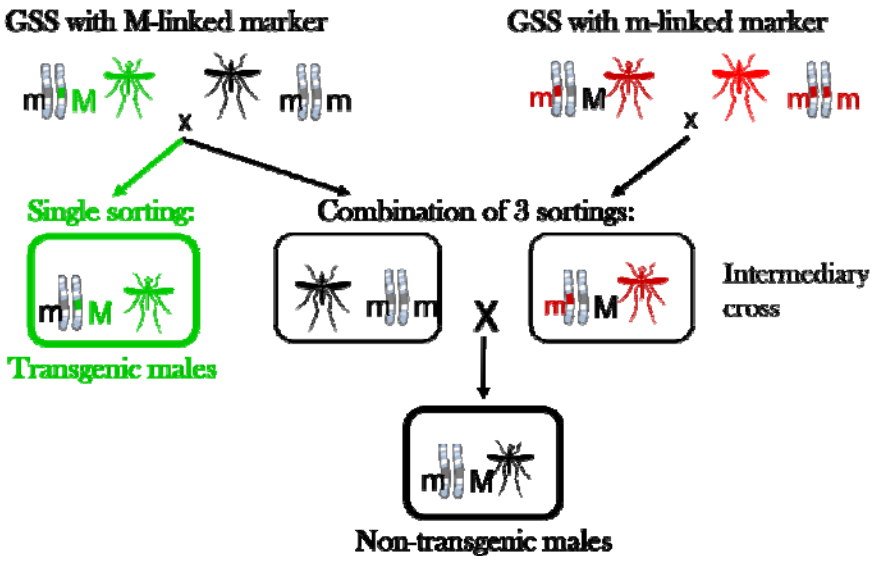

Figure 1: Combination of two GSSs for sorting non-transgenic males. Schematic representation of two sex-sorting strategies. The first (green arrow and rectangle) consists in sorting transgenic males directly from the GSS carrying an M-linked marker. The second (black arrows and rectangles) consists in using the M-linked GSS for sorting non-transgenic females and the m-linked GSS for sorting hemizygous transgenic males. These two sex-sorted populations are then crossed. In their progeny, females are transgenic while males are not. The latter can be sorted for the release of non-transgenic males.

In this work, we developed two GSSs for both *Aedes* vector species, one linked to the M-locus and another to the m-locus, using gene engineering and transgenesis. We show that these GSSs can be used separately to release transgenic mosquitoes or combined to purify large populations of wild-type males. We discuss the suitability of the method for mass-rearing and inundative releases.

Results

Obtaining four Genetic Sexing Strains

In *Ae. aegypti*, linkage of an e*GFP* transgene to the m and M loci was achieved by CRISPR-Cas9 knockin targeting the AAEL019619 gene located in the non-recombining region encompassing the sex-loci [28] (**Figure 2a-b,** see **Methods** for further details). We isolated an *Ae. aegypti* M-linked strain

that we termed Aaeg-M, and an *Ae. aegypti* m-linked strain that we termed Aaeg-m. The *GFP* fluorescence in both lines allows sex separation at the neonate stage: in Aaeg-M, males express *GFP* while females are non-transgenic (**Figure 2c**) and in Aaeg-m, males express one copy of the *GFP* transgene, while females express two copies resulting in brighter fluorescence (**Figure 2d**). Both lines were backcrossed 7 times to a Brazilian genetic background (Bra). In *Ae. albopictus*, M-linkage of fluorescence markers was achieved by *piggyBac* homing stimulated by sequences derived from the *Nix* gene and cloned in the *piggyBac* transgenesis plasmid (**Figure 2e,** see **Methods**). This approach yielded at least eight lines with tight M-linkage out of about 60 screened *piggyBac* insertions. Among the tightly M-linked *Ae. albopictus* strains, we selected one expressing YFP, that we termed Aal-M. To obtain a second sexing strain with m linkage in the absence of knowledge of the *Ae. albopictus* m locus sequence, we screened >120 *piggyBac* random insertions (**Figure 2f**). The best m-linked insertion line that we obtained showed a recombination frequency of 0.1%. Its transgenic cassette harboured a *Cas9* transgene associated with a *DsRed* fluorescence marker and was flanked by lox sites. A *Cas9* transgene being undesirable in a sexing strain, we excised it using CRE recombinase and replaced it with an *eGFP* transgene. This *Ae. albopictus* m-linked strain was termed Aal-m. Sequencing of the transposon's flanking genomic sequence indicated that it had landed into a highly repeated region; hence, its exact genomic location could not be identified (see **Methods** and **Supplementary Data 1**). Strickingly, no or weak M-linkage was obtained with these *piggyBac* constructs devoid of *Nix* sequence. Both *Ae. albopictus* transgenic lines show a clear sex-separation pattern in COPAS analyses (**Figure 2g-h)**. All four lines were fluorescence-sorted and screened at each generation. In the Aaeg-M line, a single recombination event was observed after 15 generations (>10,000 individuals screened in total). Consequently, we estimated that the Aaeg-M and Aaeg-m lines recombine at approximately 0.01%. In Aal-M, four recombinant individuals were observed in the tenth generation after successively screening a total of >50,000 individuals. These recombinations are likely to have arisen from a single event, suggesting that recombination is extremely rare in the Aal-M strain as well. Of note, all tested recombinants in Aal-M appeared to be sterile.

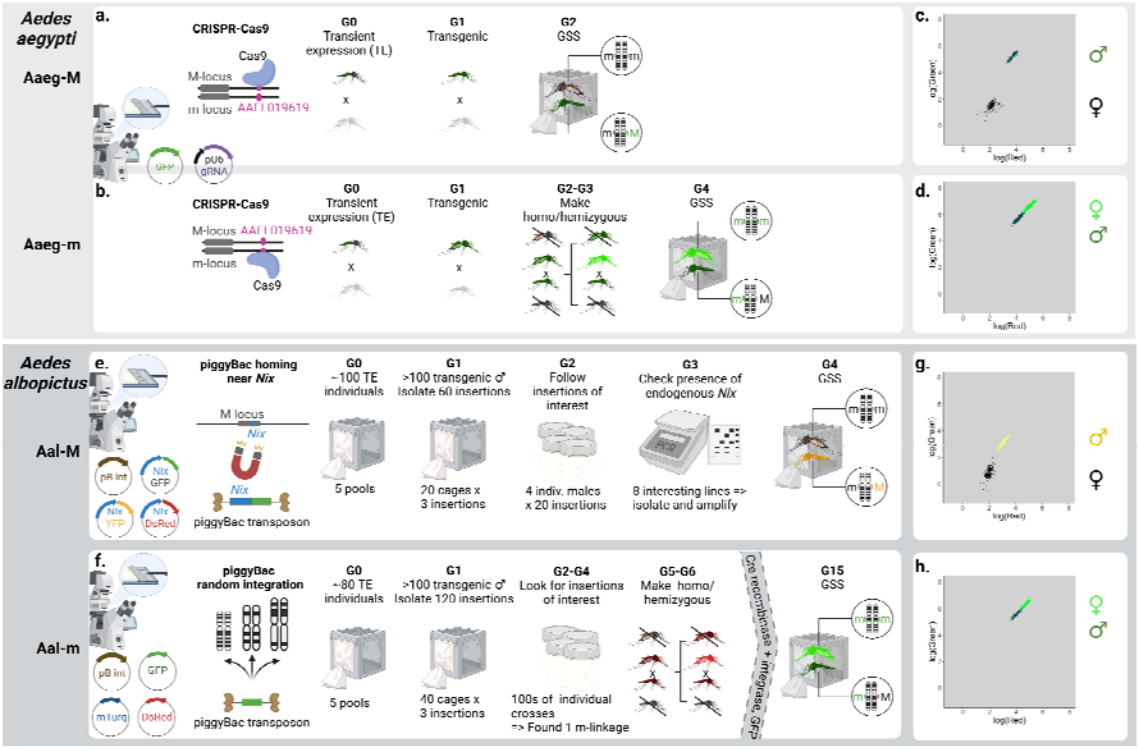

Figure 2: Schematic representation of the procedures used to obtain linkage of a fluorescence marker to the M and m-loci in *Ae. aegypti* and *Ae. albopictus.* In. *Ae. aegypti*, a single CRISPR-Cas9 target was used to obtain an M-linked strain (**a**) and an m-linked strain (**b**) in 2 to 4 generations. Both lines allow sex separation using a COPAS flow cytometer **(c-d)**. In *Ae. albopictus*, M-linkage was obtained by *piggyBac* homing near the M-locus in 4 generations, requiring screening and testing (**e**). "pB int" = helper plasmid expressing piggyBac transposase. In *Ae. albopictus*, m-linkage was obtained by *piggyBac* random integration through intensive screening over 6 generations (**f**). The resulting line was then modified using CRE-recombinase and a *GFP* expressing plasmid docking into the attP site left-over after lox cassette excision. Both Aal-M and Aal-m allow sex separation using a COPAS flow cytometer **(g-h)**.

Combining M and m-linked Genetic Sexing Strains for purifying non-transgenic males

A pilot experiment to obtain non-transgenic males using two GSS was performed in both *Ae. aegypti* and *Ae. albopictus*. In *Ae. aegypti*, 1,000 negative females from Aaeg-M were COPAS-sorted (**Figure 3a**) and crossed with 300 COPAS sorted hemizygous males from Aaeg-m (**Figure 3b**). Their progeny (Aaeg-CS) comprised 65,839 larvae, of which 32,960 were negative and supposedly males (**Figure 3c**). We COPAS-extracted 2,000 of those, all of which emerged as non-transgenic males. In *Ae. albopictus*, we applied the same crossing scheme using 850 negative females from the Aal-M strain

crossed with 250 hemizygous males from the Aal-m strain (**Figure 3d-e**). In their progeny, we obtained 24,239 Aal-CS larvae, of which 12,173 were negative and presumably males (**Figure 3f**). Of the 2,000 negative COPAS-extracted larvae, 1,468 pupae were recovered and visually verified, revealing the presence of one contaminant non-transgenic female, which is consistent with the estimated 0.1% recombination rate of the Aal-m strain.

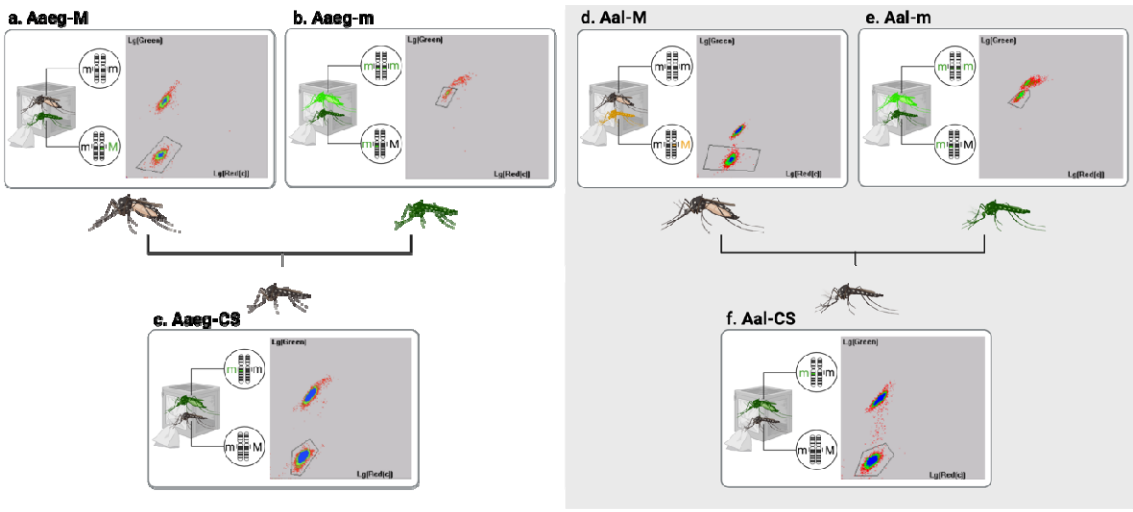

Figure 3: Successive sortings leading to purification of non-transgenic males. All panels show the fluorescence graphs log(Green) = f(log(Red)) displayed by the COPAS software. Gated regions were defined for sorting populations of interest. **a.** Sorting of non-transgenic females from Aaeg-M strain. Total larvae = 6,079. **b.** Sorting of hemizygous transgenic males from Aaeg-m strain. Total larvae = 711. **c.** Sorting of non-transgenic males in the progeny of 1,000 non-transgenic females from Aaeg-M strain crossed with 300 hemizygous transgenic males from Aaeg-m strain. Total larvae = 65,839. **d.** Sorting of non-transgenic females from Aal-M strain. Total larvae = 6,797. **e.** Sorting of hemizygous transgenic males from Aal-m strain. Total larvae = 1,960. **f.** Sorting of non-transgenic males in the progeny of 850 non-transgenic females from Aal-M strain crossed with 250 hemizygous transgenic males from Aal-m strain. Total larvae = 24,239.

Assessing the fitness of the Genetic Sexing Strains

The fitness of the selected M and m-linked lines, as well as of the intermediary colony from the sexing scheme (non transgenic females from the M-linked line crossed with hemizygous transgenic males from the m-linked line), was analysed by examining the following parameters: sex ratio, fecundity

(number of eggs per female), egg hatching rate, and survival from larva to adulthood. In *Ae. aegypti*, lines Aaeg-M, Aaeg-m and the intermediary colony from the crossing scheme (Aaeg-CS) showed similar sex ratios to the Bra WT line (**Figure 4a, Supplementary Table 1 and Data 2**). The fecundity of Aaeg-M and Aaeg-m was not different from that of Bra (WT), while Aaeg-CS had a higher fecundity (**Figure 4b**). Aaeg-M eggs hatched similarly to Bra eggs, while Aaeg-m eggs and Aaeg-CS eggs hatched significantly better (**Figure 4c**). Survival from neonate larvae to adult stages was not significantly different in Aaeg-M, Aaeg-m and Aaeg-CS compared to Bra (**Figure 4d)**. In *Ae. albopictus*, the sex ratios of the Aal-M, Aal-m and Aal-CS lines were not significantly different from that of the wild-type line BiA (**Figure 4a**). Aal-M had significantly higher fecundity than BiA (WT), while Aal-m and Aal-CS had similar fecundities to WT (**Figure 4b**). Eggs from the Aal-M and Aal-CS lines had a slightly higher hatching rate than eggs from BiA (WT), while Aal-m eggs hatched similarly (**Figure 4c**). Survival from neonate larvae to adults was marginally increased in Aal-M and Aal-m compared to BiA (WT), while it was not significantly different in the progeny of the crossing scheme (Aal-CS, **Figure 4c**).

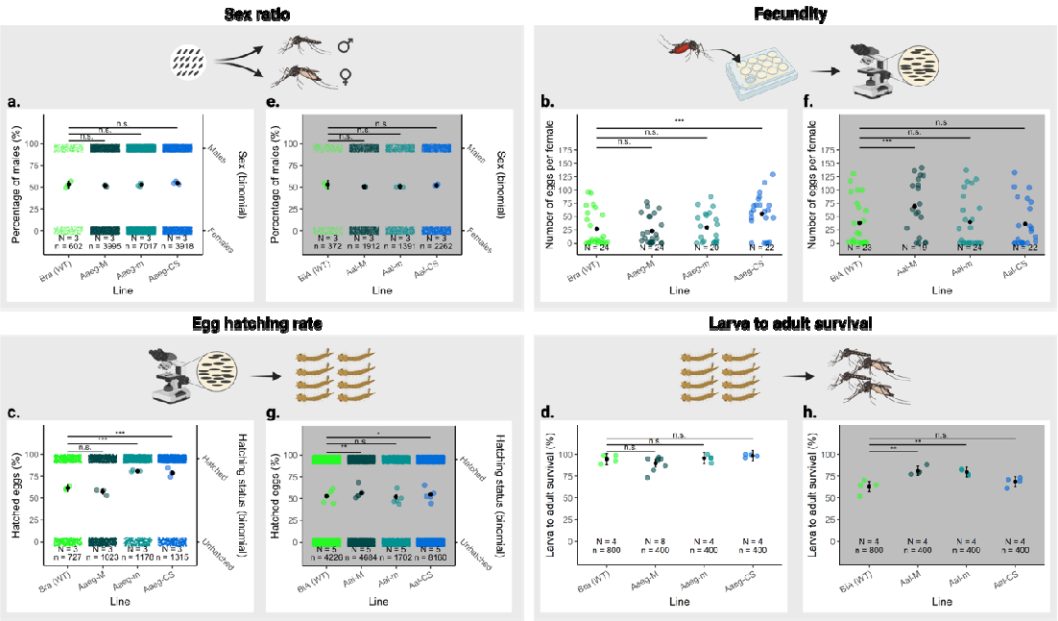

Figure 4: Fitness of the genetic sexing strains and of the intermediary colony yielding non-transgenic males. In all panels, the black dots with vertical bars represent the mean value and the 95% CI in the *Ae. aegypti*

and *Ae. albopictus* assays, respectively. The thin coloured dots in the squares above and below estimates represent individual data points (n), while the large coloured dots around estimates represent the mean values of each replicate (N). Significant differences between the lines are specified in the figure ("n.s.": no significant difference. "*": p-value < 0.1, "**": p-value < 0.01, "***": p-value < 0.001). The percentages of males were compared using a generalised linear model with a binomial distribution in the *Ae. aegypti* (**a**) and *Ae. albopictus* (**e**) lines. Comparisons of the mean number of eggs laid by an individual were analysed using a generalised linear model with a Poisson distribution in the *Ae. aegypti* (**b**) and *Ae. albopictus* (**f**) lines. Comparisons of the percentage of egg hatch were analysed using a generalised linear model with a binomial distribution in the *Ae. aegypti* (**c**) and *Ae. albopictus* (**g**) lines. Comparison of survival from neonate larvae to adult stages were analysed using a linear model with the assumption of residual normality in the *Ae. aegypti* (**d**) and *Ae. albopictus* (**h**) lines.

Assessing the fitness of the males to be released

In an operational SIT approach, transgenic males from the M-linked lines or non-transgenic males from the crossing scheme would be intended for release. With this aim in mind, we assessed their fitness through competitiveness assays, flight tests and two-week survival tests under laboratory conditions. In the competitiveness assay between transgenic and non-transgenic males in *Ae. aegypti*, Aaeg-M performed equally to Bra (WT) and Aaeg-CS (**Figure 5a, Supplementary Table 1**). In *Ae. albopictus*, given the higher hatching rate and fecundity of Aal-M compared to BiA (WT) and Aal-CS, equal competitiveness would result in higher than expected percentages of Aal-M in both competitiveness assays. Aal-M showed a 0.59 competitiveness compared to BiA (WT) and a similar competitiveness compared to Aal-CS (**Figure 5b**), which means that both Aal-M and Aal-CS have a reduced competitiveness as compared to WT. In flight tests, *Ae. aegypti* transgenic males and non-transgenic males from the crossing scheme performed as well as control males, while in *Ae. albopictus*, the escape rates of Aal-M males were marginally lower and those of Aal-CS males marginally higher than those of WT, with no significant difference (**Figure 5c-d**). Adult survival was monitored for two weeks and no significant differences were observed between transgenic males, non-transgenic males from the crossing scheme, and males from their respective WT lines (**Figure 5e-f**).

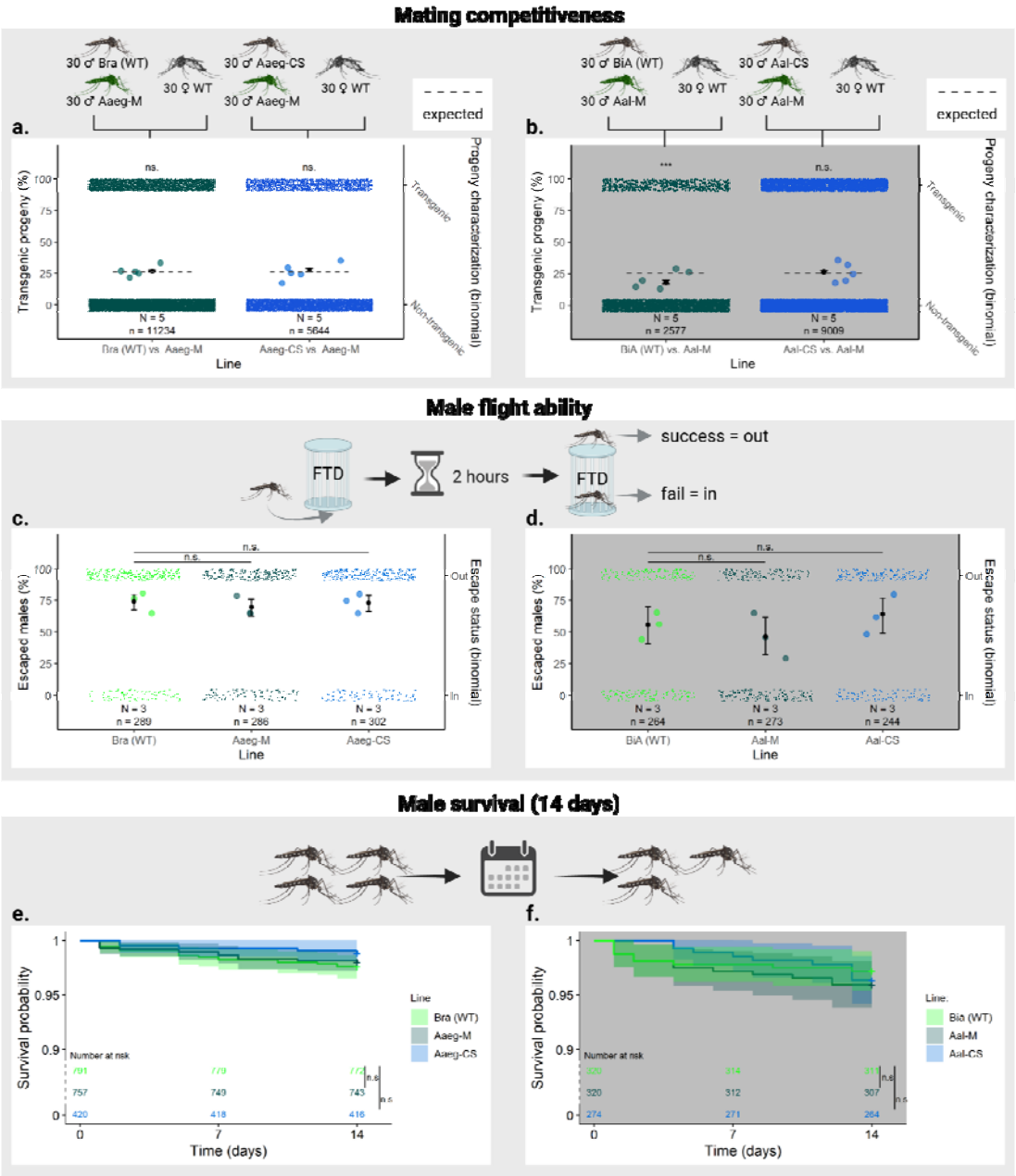

Figure 5: Fitness of transgenic and non-transgenic males as compared to wild type. Black dots with vertical bars represent the mean value and 95% CI in the competitiveness and male flight ability assays. The thin coloured dots in the squares above and below the estimates represent individual data points (n), while the large coloured dots around the estimates represent the mean values of each replicate (N). Significant differences between the lines are indicated in the figure ("n.s.": no significant difference. "*": p-value < 0.1, "**": p-value < 0.01, "***": p-value < 0.001). The competitiveness of males of the Aaeg-M line relatively to Bra (WT) or Aaeg-

CS in *Ae. aegypti* (**a**) and the competitiveness of males of the Aal-M line relatively to BiA (WT) and Aal-CS in *Ae. albopictus* (**b**) were compared by measuring the number of transgenic and non-transgenic neonate larvae in the progeny of each of the N = 5 replicates. Results were compared to the percentage expected if transgenic males were as competitive as non-transgenic males (dashed line) and were analysed using a generalised mixed-effects model with a binomial distribution and replicates as a random effect. The flight ability of transgenic and non-transgenic males compared to the wild-type in *Ae. aegypti* (**c**), and *Ae. albopictus* (**d**) was tested by N = 3 replicates in a reference flight test device ("FTD"). The results were analysed using a generalised mixed-effect model with a binomial distribution and replicates as a random effect. Survival of adult males 14 days after emergence was monitored in *Ae. aegypti* (**e**) and *Ae. albopictus* (**f**) lines. N=4 replicates were performed for each line. All values being above 90%, the y-axis is discontinuous between 0 and 90% for better visualisation. Male survival at 7 and 14 days was compared using the log-rank test and neither comparison was significantly different.

Determining the ideal COPAS-sorting speed

To assess the reliability of COPAS-sorting for high-throughput mass production of males in a mosquito production facility, we evaluated the contamination rate as a function of sorting speed. A pool of 10,000 Aaeg-CS neonate larvae (fluorescent females + non-fluorescent males) was repeatedly COPAS-sorted and we measured the male recovery rate and the female contamination rate at different sorting speeds. Sorting at a given speed was repeated N = 3 times. We observed that the percentage of recovery decreased from 91.5 ±1.4% to 26.9 ±1.9% when increasing the sorting speed from 6 to 300 larvae / sec, following a degree-two polynomial regression model (R^2 = 0.983), while contamination remained absent below 300 larvae/sec (**Figure 6**). We estimate that an operational speed for sex sorting would be around 60 larvae/sec (which corresponds to 200 larvae/mL in the reservoir water) with a mean recovery rate of 69.6 ±1.1% and no female contamination detected among more than 6,500 sorted males. At this speed, a small COPAS reservoir (250mL) holds 50,000 larvae that are sorted in <14 minutes, yielding approximately 17,400 ± 270 males (considering a 50:50 sex ratio). Consequently, 100,000 males could be sorted in less than 1.5 hours. The same test run was repeated with *Ae. albopictus* neonate larvae and gave comparable results (**Figure 6**).

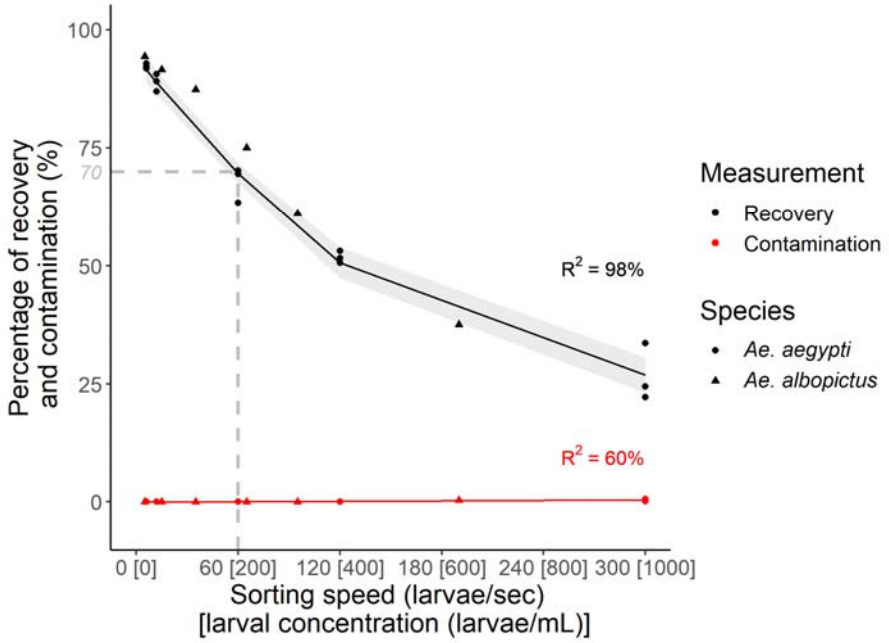

Figure 6: Evolution of male recovery and female contamination rates as a function of COPAS sorting speed. 10,000 *Ae. aegypti* neonate larvae from the crossing scheme were COPAS-sorted at different concentrations, resulting in different sorting speeds. Percentage of recovery (black dots) and percentage of contamination (red dots) were measured on three replicates for each sorting speed. Recovery values were fitted using a degree-two polynomial regression ($R^2 = 0.983$) and contamination values were fitted using a linear regression ($R^2 = 0.556$). The model predictions were plotted by black and red lines, respectively, with 95% confidence intervals ribbons. The same experiment was repeated with 4,000 *Ae. albopictus* neonate larvae from the crossing scheme. In this species, percentage of recovery (black triangles) and percentage of contamination (red triangles) were measured on a single replicate for each sorting speed.

Discussion

In this paper, we present the first genetic sexing strains (GSSs) for *Ae. aegypti* and *Ae. albopictus* based on m/M-linked transgenic fluorescent markers. We show that the M-linked Aaeg-M and Aal-M strains can be used alone to sort transgenic neonate larvae and that the M- and m-linked strains of each species can also be combined to produce a non-transgenic male population by two rounds of sex-

sorting (of the parental strains and of the cross' offsprfing). In *Ae. aegypti*, transgenic males from the M-linked strain and non-transgenic males from the crossing scheme were found to be as fit and competitive as wild-type males of the same laboratory strain. In *Ae. albopictus*, a mild competitiveness reduction was observed in Aal-M transgenic males as well as in Aal-CS non-transgenic males. At a speed of 60 larvae/second using a COPAS device, we estimate that 100,000 males can be obtained in under 1.5 hour with the current settings, recovering 70% males and with a female contamination of 0.01-0.1%.

Unexpectedly, we observed rare recombination events between the transgene and the M-locus in the Aaeg-M line after screening tens of thousands of larvae. In this line, the transgene is located approximately 9 Mbp from *Nix*, which remains well within the presumed non-recombining region [28]. Compared to the probability of recombination between two markers separated by the same distance at regular loci (3.4-4.7% according to the latest genomic information [25]), these results confirm that recombination is strongly reduced in this region, but not totally suppressed. Although not yet observed, the Aaeg-m line is expected to recombine at the same frequency as Aaeg-M, as both transgenes are inserted in the same target gene at a similar distance from their respective sex loci. The recombination events imply that these GSSs will require regular purification of the parental colonies to avoid an accumulation of recombinant individuals. Of note, such recombination events are easy to spot in single-sex batches of pupae due to size dimorphism and protandry, which will facilitate regular quality control of each strain.

Although our attempts at knocking-in a GFP marker into the large intron of *Nix* failed (not shown), better GSSs may be constructed by targeting regions closer to or within the sex loci, especially in *Ae. aegypti* which benefits from a fully assembled genome. In *Ae. albopictus*, rare recombination events were also found in Aal-M amongst more than 10,000 individuals screened over 10 generations. For more accurate genome editing, better assembly of the first chromosome would be required in this species. The lack of genetic stability under mass-rearing conditions and the appearance of aberrant sex recombinants have already been reported in the development of GSSs in other insect model organisms [29]. Alternatively, the creation of viable homozygous chromosomal inversions overlapping the sex-

determining locus and the fluorescent transgene could help suppress crossing-over [30]. Finally, we have recently reported that transgenic *Ae. albopictus* pseudo-males masculinised by ectopic expression of *Nix* can be used as Aal-M GSS in which the fluorescent marker will show an absolute linkage to sex [24]. This type of GSS is particularly interesting for the production of non-transgenic males, since in this case the wild-type females derived from the Aal-M line and used in the crossing scheme are both non-transgenic and devoid of any recombinant contamination.

Compared to current sex-separation methods in *Aedes* mosquitoes [8,10,12], these GSSs allow a higher male recovery than any other sorting methods based on pupal size (about 70% *vs*. 30%). The female contamination rate (0.01 to 0.1% depending on recombination events) is higher than with the Verily's multi-step sorter [8], but much lower than with manual[12] or automated[10] glass sorters. Our system offers a sorting speed similar to that of the automated glass sorter, which is higher than that of manual size sorters and multi-step sorters. Compared to all of these currently available methods, the main advantage of our GSSs is to allow sorting at the earliest stage of development, which results in significant savings in space, time and cost otherwise spent on rearing unnecessary individuals. Moreover, reducing the number of females reaching the pupal stage in the mass-rearing facility decreases the biting nuisance for the workers, a problem frequently observed with pupal sorting methods, due to early adult emergences.

Additionally, we propose a crossing strategy that proved to be effective in obtaining non-transgenic males. Compared to sorting pupae by size with glass plates or metal sieves, it has the advantage of automating all sorting steps and achieving much lower female contamination rates. Although it requires the rearing of two strains plus the sorting of males in the mass-rearing facility, this only represents the maintenance of approximately 2% of additional mosquitoes. In comparison to the savings achieved in rearing the released mosquitoes, this is negligible.

Although this work has focussed on the production of *Aedes* males for SIT, the same procedures for the production of transgenic or non-transgenic male could be directly applied in other mosquito control approaches such as IIT, RIDL, pg-SIT or other genetic control methods currently under development.

Of note, GSSs with an m-linked fluorescence marker show less obvious sex separation than M-linked GSSs. However, contamination of the sexing cross with females homozygous for the transgene only results in transgenic progeny that is subsequently eliminated when sorting the non-transgenic males. The release of non-transgenic males from crossbreeding schemes may in fact be more appropriate than the release of transgenic males, since GMO mosquito releases face public opposition and regulatory constraints [31]. Therefore, costs related to public information and consultation campaigns, stakeholder engagement and compliance with regulatory processes may be reduced. These aspects need to be taken into account as in the past, lack of information and consultation has led to such strong public opposition that some release programmes have had to be cancelled [31]. Of note, recombination in our GSSs would result in contamination of released males by non-transgenic irradiated females at a rate equal to the recombination rate; however, this release of sterile females would still be much lower as compared to current methods.

We found COPAS errors during the purification of non-transgenic males were extremely rare in our pilot experiments. However, such contaminations may occur due to occasional technical problems, and would result in the release of a small percentage of transgenic mosquitoes amidst the non-transgenic. This should be absolutely avoided in settings where GMOs are banned. An additional quality control step to eliminate any residual GFP fluorescent larvae from the sorted larvae could be achieved by a second COPAS run. It could also be achieved by a visual verification of the sorted larvae under a fluorescence microscope, as GFP positives are easy to spot and eliminate, even among many wild-type larvae.

In conclusion, the four GSSs developed in this work represent a very promising tool for improving the cost-efficiency of *Aedes* mass-rearing in genetic control programmes. According to our laboratory pilot tests, a facility harbouring two COPAS machines could produce 1,000,000 male larvae per day in a 7.5-hours regime. Considering that no females are reared, space constraints would be eased. Moreover, this technique would also reduce the production costs associated with low or no female contamination, facilitating compliance with local regulations. Our results suggest that COPAS sorting of our GSSs would allow a rapid, economical, and safe mass-production of male mosquitoes for SIT

and other genetic control methods, with flexible up-scaling possibilities according to local needs, resources and regulations.

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Author contributions

C.L., T.B., J.B. and E.M. designed research. A.F. contributed genomic data. C.L., M.B., R.P.O. and E.M. performed research. C.L., R.P.O., R.B. and A.F. analysed data. R.B. contributed analytic tools. C.L. wrote the paper with inputs from all authors.

Competing interests statement

The authors declare no competing interests.

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Methods

Mosquito rearing

The *Ae. albopictus* wild-type colony (called BiA) was established from larvae collected in Bischheim (France, 48.36°N 07.45°E) in 2018. *Ae. aegypti* from the Bangkok genetic background obtained from MR4-BEI were used for molecular cloning and genetic engineering. *Ae. aegypti* strain BgR9, derived from Bangkok, and expressing *Cas9* under the control of the Exuperentia promoter and a *DsRed* reporter gene under the *Ae*PUb promoter was used for CRISPR/Cas9 knock-in experiments. The Bra *Ae. aegypti* strain originated from Juazeiro, Brazil and was provided to the IPCL in 2012 by Biofabrica Moscamed, a collaborative centre of the IAEA on the development of the SIT against mosquitoes. It was used for backcrossing the sexing transgenes.

Mosquitoes were maintained in standard insectary conditions (25-28°C, 75-80% relative humidity, 14-hr/10-hr light/dark cycle). Larvae were reared in 35 cm square pans in 1 L distilled water and daily fed ground TetraMin fish food. Adult mosquitoes were kept in 16x16x16-cm cages and provided a 10% sugar solution. Females were blood-fed on anesthetised mice. Eggs were laid on humid kraft paper and hatched or dried after 3 days.

Plasmid construction

Plasmid construction was done using standard molecular biology procedures including PCR amplification of genomic DNA using Phusion™ High-Fidelity DNA Polymerase (F530, Thermo Scientific, France), cloning with NEBuilder® HiFi DNA Assembly (New England Biolabs, France), GoldenGate cloning in destination backbones using Anza 36 Eco31I restriction enzyme (Thermo Fisher Scientific, France), transformation in competent *Escherichia coli* bacteria, and Miniprep or Endo-Free Midiprep plasmid purifications (Macherey Nagel, France). For CRISPR/Cas9 knock-in in the *Ae. aegypti* M and m loci, we used plasmid pX4 and pX3 as repair donors, respectively (**Supplementary Data 3,** Addgene #183903 and #183904). For *piggyBac* integration near the *Ae. albopictus* M locus yielding the Aal-M strain, we used plasmid ppBAalbNixE1E3E4 PUb-YFP (Addgene #173666 described in [24]). For random *piggyBac* insertion near the m locus, we used plasmid ppBExu-Cas9-sv40 (Addgene #183905), the lox cassette of which (encompassing *Cas9* and PUb-

DsRed) was subsequently excised from the genome by injection of a plasmid expressing Cre recombinase under the control of the PUb promoter, and replaced by insertingplasmid pENTR-attB-PUb-GFP (Addgene #183911) into the *attP* site by co-injecting it with a plasmid expressing integrase from phage PhiC31 (Addgene #183966).

Microinjection

Injection mixes were composed of 400ng/µL of DNA in 0.5x PBS. *piggyBac* injection mixes were prepared as described in [18]. For CRISPR-Cas knock-ins, the initial mix injected into the BgR9 *Ae. aegypti* Cas9-expressing line comprised 84 µM gRNAs, 100 ng/µL repair plasmid and 2µM Scr7. Given the very low number of transgenics obtained (1 individual out of >20,000 screened larvae), we later injected the repair donor plasmid (190 ng/µL) with 5µM Scr7 and three plasmids expressing different gRNAs under the control of different *Ae*U6 promoters (70ng/µL each plasmid). This method gave significantly higher knock-in rates (25 GFP positive larvae out of about 4000 screened).

Embryo microinjection was performed essentially as published using a Nikon Eclipse TE2000-S inverted microscope, an Eppendorf Femtojet injector and a TransferMan NK2 micromanipulator [18] with the following modifications: eggs were hatched 3-8 days post-injection and neonate larvae were screened under a Nikon SMZ18 fluorescence microscope, only larvae showing transient expression of the reporter gene were retained for subsequent outcross.

***Ae. aegypti* transgenesis**

For targeting the sex loci, we exploited the work of Fontaine *et al.* showing a cluster of markers inside a non-recombining 63 Mbp region encompassing the sex loci with high male-female genetic differentiation and male heterozygosity consistent with Y chromosome-like null alleles [28] (**Supplementary Table 3**). DNA sequences of these markers with putative Y-linked-like null alleles were blasted on the assembled AegL5 genome [25] to identify a 4.9 Mb region extending from 160,092,339 bp to 165,014,624 bp on chromosome 1 in the AegL5 assembly. Within this non-recombining region we picked the gene AAEL019619, located ~9Mbp away from *Nix*. We used the

CRISPR/Cas9 system to knock-in a 2.3 kb fluorescence marker cassette (*eGFP* marker under the *Ae. aegypti* poly-Ubiquitin promoter - PUb) flanked by 850-960 bp homology arms either within the fifth, 9 kb-intron of this gene (pX3 construct), or to create a synthetic intron within exon 5 (pX4 construct). We obtained a first M-linked strain for *Ae. aegypti* following the injection of 200 eggs of the BgR9 strain, which expresses *Cas9* under control of the *Exuperentia* promoter, with the pX4 repair plasmid and two synthetic gRNAs (GATGAATCATGGGGCGCCT[GGG] and GATTCATCAATCAACGGAG[CGG], brackets indicate PAM). About 30 G0 larvae showing transient GFP expression were crossed *en masse* to an excess of wild-type mosquitoes. Out of > 20,000 G1 larvae screened, a single transgenic male larva was found, raised to adulthood, and crossed to WT females. Its progeny was composed of 100% eGFP positive sons and 100% eGFP negative daughters, indicative of M linkage. We termed this *Ae. aegypti* M-linked strain Aaeg-M. Upon injection of 600 BgR9 eggs with the pX3 repair plasmid and a mixture of three plasmids expressing gRNAs (GTGGCATAGCGCCGTGTGGA[GGG], GTCTTAAATGAAAGAGGCG[AGG], GTATCATGCGTATTGCGAG[AGG]) under the control of three different U6 promoters (gRNA cloning vectors: **Supplementary Data 3**), 43 larvae with transient eGFP expression were recovered in G0. Resulting 20 males and 20 females were crossed *en masse* to adults of the opposite sex. 25 transgenic G1 larvae were obtained, 10 of which were males carrying an M-linked insertion, 4 were males carrying an m-linked insertion and 11 were females carrying an m-linked insertion. Proper insertion site in AAEL019619 was confirmed by PCR in all tested individuals, which revealed that in some of these mosquitoes the whole repair plasmid had integrated. From a single male devoid of the plasmid backbone, an *Ae. aegypti* m-linked strain named Aaeg-m was established and further characterized. Both Aaeg-M and Aaeg-m lines were backcrossed into a Brazilian (Bra) genetic background for 7 generations.

***Ae. albopictus* transgenesis: obtaining M-locus linked strains**

Following several unsuccessful attempts to knock-in a fluorescence marker near the *Ae. albopictus Nix* gene using the CRISPR/Cas9 system, we took advantage of the frequent spontaneous insertion near the M locus of *piggyBac* transposon constructs carrying *Nix* DNA sequences that we previously

reported [24]. In the course of several transgenesis experiments, using *piggyBac* constructs carrying *Nix* sequences, we obtained 12 independent transgenic insertions with apparent M-linkage of the transgenesis reporter (100% fluorescent males) in addition to several *Nix*-expressing masculinized female lines and to non-M linked, non-masculinizing insertions. From the third generation on, these lines were amplified to confirm their M-linkage and estimate their recombination rate. Six lines showed no recombination out of >700 individuals screened in each line until the sixth generation (**Supplementary Table 4**). Most other M-linked lines also showed 100% fluorescent males but contained additional, non-M-linked multiple insertions and were discarded. Based on line fitness and COPAS profiles, we selected an YFP line termed Aal-M for further work as an *Ae. albopictus* M-linked strain. Additional M-linked lines marked with OpIE2-GFP or Pub-DsRed showing no recombination to date, carrying a docking *attP* site for subsequent transgenesis, and representing additional GSSs are also being maintained to date but were not further characterized in detail.

***Ae. albopictus* transgenesis: obtaining an m-locus linked strain**

We first attempted to obtain an m-linked fluorescence marker by screening through >120 random *piggyBac* insertions as described. For this, we screened our existing collection of *Ae. albopictus* transgenic lines for m/M-linkage and constructed an additional library of *piggyBac* constructs expressing various fluorescence proteins (eGFP, mTurquoise2, YFP or DsRed) under the control of promoters showing distinct expression patterns (3xP3, OpIE2, *Ae. aegypti* PUb, *Drosophila melanogaster* actin5C). By performing multiplex injections of these *piggyBac* plasmids together, we obtained lines carrying up to six distinct insertions as indicated by their colours and patterns of fluorescence. Out of 40 independent founder transgenic individuals screened (most of which carrying multiple insertions), only two insertions appeared m-linked: one carried an OpIE2-mTurquoise2 marker gene with a recombination rate of about 3%, and the other, called m-albR9, carried a *Cas9* transgene with a *DsRed* reporter gene and showed a recombination rate of 0.1%. We exploited the latter, in which transgenes are flanked by two *lox* sites adjacent to an *attP* docking site. We injected m-albR9 eggs with a plasmid encoding Cre recombinase to excise *Cas9* and *DsRed* transgenes, as *Cas9* is undesirable in a GSS. From successfully excised individuals, we established an m-linked *attP*

docking line, mX1, carrying no further transgene. In a second step, an *attB*-containing plasmid carrying a PUb-eGFP marker cassette was integrated into the *attP* site. The m-linkage of this new strain was verified by crossing eGFP males to WT females and screening their offspring. The new *Ae. albopictus* m-linked line, named Aal-m was made hemi/homozygous and amplified.

Automated larva sorting

Automated sorting of neonate larvae depending on their fluorescence level was performed using a large-object flow cytometer named Complex Object Parametric Analyzer and Sorter (COPAS SELECT; Union Biometrica, Belgium) as published [16], with the provided Biosort software. Sorting accuracy was controlled under a Nikon SMZ18 fluorescence microscope.

Analysis of COPAS outputs

To determine the exact number of larvae in COPAS clusters, we built a clustering algorithm based on manual outlier screening of particle size and fluorescence distribution, and an automated clustering algorithm. All data handling and statistical calculations were performed using the R statistical software version 4.1.0 [32]. All R scripts used for this project can be found in our GitHub repository (https://github.com/Celia-Lutrat/Lutrat-et-al_2022_GSS). Briefly, COPAS raw data were filtered by the user via the visual examination of the individuals' size measurements (i.e., "log(EXT)" and "log(TOF)") to remove outliers (e.g., egg debris and dust). A second filtering was performed on the two first axes of a Primary Component Analyses (PCA) applied to the size variables (EXT and TOF). A third manual filter was applied by examination of the individuals' fluorescence (i.e., "log(first fluorescence)" and "log(second fluorescence)") . A final filtering was performed on the two first axes of a PCA applied to fluorescence variables. PCA analyses were performed using the R function 'prcomp' from the package 'stat' [32] and the PCA outputs were extracted using the R package 'factoextra' [33]. Filtered data were clustered using the R function 'kmeans' from the R package 'stat' [32]. Data were plotted using the R packages 'ggplot2' and 'ggpubr' [34,35].

Targeted sequencing method

Genomic DNA was extracted using NEB Monarch HMW DNA extraction kit (T3060, New England Biolabs, France) according to manufacturer instructions with the following modifications: after addition of isopropanol, precipitated DNA was caught directly on a pipette tip and transferred to a new tube containing 550 µl wash buffer and then to a final tube containing 70% ethanol. DNA was stored in 70% ethanol at 4°C until use. On the day of use, ethanol was removed and DNA was resuspended in water at 37°C with 500rpm agitation and gentle pipetting. DNA concentration was then measured using a Nanodrop One device (Ozyme, France).

Sequencing method was adapted from the Nanopore Cas9 Targeted Sequencing (nCATS [36]). gRNAs were selected in the known transgene region, with their PAMs towards the unknown genomic sequence to be determined. This orientation ensured preferential ligation of Nanopore adapters on DNA strands of interest.

Adapter ligation and library preparation were performed using the LSK109 kit (Oxford Nanopore Technologies, UK) according to manufacturer instructions. Library was loaded on Flongle flow cells. Sequencing, basecalling and alignment of reads to the transgenesis plasmid were performed using the provided MinKNOW v. 21.06.10 software (Oxford Nanopore Technologies, UK). Reads that showed homology to the transposon ends were then aligned to the transposon sequence using ClustalW on Geneious software v9.1.8 [37] and one or several consensus sequences were determined. Finally, the consensus sequences were compared to genomic data using the online NCBI BLAST service [38].

This method allowed us to determine the insertion site of the Aal-m transgene. We obtained only two low-quality reads extending by about 20 kb into the genomic sequence adjacent to the transgene, from which we designed a series of PCR primers. Sanger sequencing of PCR products spanning the transposon/genomic junction allowed us to refine 1647 bp of the flanking sequence, provided in **Supplementary Data 1.** Analysis of this sequence using NCBI BLAST showed only partial alignment to sequences of the AalbF2 genome [39], presumably corresponding to a repetitive element, suggesting that the m-linked region where the transgene inserted is not represented in the available genome

assemblies.

Assessment of lines' fitness

In transgenic lines, the sex ratio was measured by COPAS counting of the larvae of each fluorescence. In non-transgenic lines (BiA and Bra), we reared samples to the pupal stage and counted males and females under a binocular microscope based on genitalia examination. N=3 replicates were made for all lines.

For fecundity, males and females were allowed to mate for several days. Cages were then offered a blood meal and engorged females were selected and placed in a separate cup. After 2 days, engorged females were shortly anesthetised using CO_2 and placed individually in 24-well plates coated with wet filter paper. After 2 days, females were released and each well was photographed under a Zeiss SteREO binocular microscope. Eggs laid by each female were counted using the ImageJ software [40].

Egg hatching rate was measured on egg samples collected from N=3-5 cages of each line, photographed under a Zeiss SteREO binocular microscope and counted using the ImageJ software [40]. Eggs were immersed in water, placed into a vacuum chamber for 20 to 30 minutes and allowed to hatch overnight. The following morning, larvae were counted by COPAS.

Larva to adult survival was measured by sorting N=4-8 samples of 100-200 neonate larvae by COPAS and rearing them to adulthood. The number of adults emerging from each batch was counted manually.

Assessment of males' fitness

Males' competitiveness was assessed by placing 30 transgenic males with 30 non-transgenic males in a cage with 30 females and measuring the proportion of transgenic progeny in their offspring. If both types of males are of similar fitness, it is expected that the progeny would be composed of ~25% transgenic individuals, corrected by the line sex ratio (transgenic fathers being hemizygous). Relative competitiveness was estimated by comparing the observed proportion of transgenic progeny to the

theoretical value in N=4 or N=5 replicates.

Males' flight ability was assessed using a flight test device (FTD) as described [41]. Tests were performed on pools of about 100 males with N = 3 replicates for each condition.

Males' 2-week survival was measured by counting the daily number of deaths in N=4-8 cages of ~100 males of each line for 14 days.

Statistical analysis

All data statistical analyses were performed using the R statistical software version 4.1.0 [32], the R scripts used for this study can be found in our GitHub repository (https://github.com/Celia-Lutrat/Lutrat-et-al_2022_GSS).

The difference of sex ratio and egg hatching rate between the lines of each species was modelled using the generalised model with binomial distribution and residuals distribution normality assumption (R function 'glm' [32]), and pairwise comparison of the lines was tested using a Tukey HSD test (R function 'glht' [42]). The difference in fecundity (number of eggs laid per female) between the lines of each species was modelled using a generalised model with Poisson distribution and residuals distribution normality assumption (R function 'glm'), and a pairwise comparison of the lines was tested using a Tukey HSD test (R function 'glht'). The differences in larval survival between the lines of each species was modelled using linear models with residuals distribution normality assumption (R function 'lm' [32]), and tested using a Tukey HSD test (R function 'TukeyHSD' [32]). The difference in male competitiveness and flight ability between the lines of each species was modelled using the generalised mixed-effect model with binomial distribution and residuals distribution normality assumption (R function 'glmer' from the package 'lme4' [43]), where technical replicates were set as random factor. Pairwise comparisons between lines were tested using a Tukey HSD test (R function 'glht'). Male adult survival during the first 14 days was modelled using Cox regressions and Kaplan Meir formula (function from the R packages 'survminer', 'survival' [44–46]). Differences in survival at 7 and 14 days were modelled using linear models with residuals distribution normality assumption (R function 'lm') and tested using a Tukey HSD test (R function 'TukeyHSD'). Model performances

were assessed using the R function 'check_model' from the package 'performance' [47]. The packages used, data handling procedures, models structure, fit and performances can be found in **Supplementary Data 2**.

Data availability

All plasmid sequences are available in **Supplementary Data 3.** Plasmids are available from Addgene under reference numbers #173666, #183903, #183904, #183905, #183911, #183912, #183913, #183914 and #183966. Mosquito strains are available upon request from EM. All data needed to evaluate the conclusions in the paper are present in the paper and/or in the **Supplementary Information.**

Animal care

Animal care was in accordance with CNRS guidelines and approved by the CREMEAS ethics committee.