



Generating cisgenic sexing strains in insect pests



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Insect pest population control via sterile insect technique markedly benefits from separation by sex prior to release. To simplify this process, traditional genetics has been deployed to develop genetic sexing strains (GSSs) for several disease vectors and agricultural pests of vast economic significance, although very few are applied in the field due to associated fitness costs and instability. In this study, we generated a method to engineer cisgenic GSS (CGSS) in insects. We use CRISPR/Cas9-mediated homology-directed repair to seamlessly translocate a sex-specific alternatively spliced intron into a dominant phenotypic gene generating a genetically stable strain that enables sex-sorting by eye. To achieve this feat, we use *Ceratitis capitata* as our model and relied on the sex-specifically spliced intron of its endogenous *transformer* gene, which we seamlessly inserted a copy into the pupal colouration *white pupae* gene. This minimal modification resulted in the generation of a homozygous strain we term IMPERIAL that was genetically and phenotypically stable where all female pupae are brown while male pupae are white with overall good fitness. By minimally editing the genome, our novel CGSS approach can be applied to other pests that may aid more efficient and economically suitable pest control.

Genetic sexing strains (GSSs) have been developed in multiple insect species of economic significance to allow easier male and female separation necessary for efficient population control. Specifically, GSSs are used within sterile insect technique (SIT) programmes, which work via frequent releases of sterilised insects into the wild for temporary population control^{1–3}. Male-only releases, aided by GSS implementation, are associated with wider released fly dispersal as well as their mating frequency with wild females, thus enhancing SIT success^{4–6}. Vast efforts have focused on GSS generation in mosquito disease vectors and agricultural fruit fly pests to avoid labour-intensive sex-sorting by eliminating the females from the released population early in the life cycle^{7–14}.

The traditional GSS approach requires two key attributes: a selectable marker and a Y-chromosome or male-determining locus linkage from which it needs to be expressed⁴. A primary example of a traditional GSS is the VIENNA 8 strain developed in the tephritid fruit fly pest *Ceratitis capitata* (Mediterranean fruit fly or medfly), which poses an increasing threat to the agricultural industry with its expanding global distribution and vast host range^{15,16}. VIENNA 8, similarly to its predecessor VIENNA 7, relies on a radiation-induced simultaneous translocation of genes involved in pupal colouration, *white pupae* (*wp*), and heat tolerance *temperature-sensitive lethal* (*tsl*) genes, onto the Y-chromosome^{17–19}. Whilst the former,

wp, has been characterised in multiple tephritids, the latter, *tsl*, has been recently identified in *C. capitata*^{16,17}. As the VIENNA 8 strain has a *wp* and *tsl* double-mutant background, the white-pupaed females die upon embryonic heat exposure, whilst the brown-pupaed males persevere into adulthood^{4,20}. Across the *Tephritidae* family, such traditional GSSs have been successfully developed in multiple species, although only those developed in *C. capitata* and *Anastrepha ludens* have been implemented on a SIT facility scale^{21,22}. The other similar GSSs, such as those built in *Bactrocera dorsalis* and *Zeugodacus cucurbitae*, based on *wp*, have, however, not reached the same scalability, despite being tested on an SIT facility level^{8–10}. Alternatively, traditional GSSs may be dependent on genes involved in eye pigmentation, such as in *Aedes aegypti*²³.

In parallel to similar GSSs in other tephritid species, the VIENNA 8 strain is infrequently susceptible to phenotype loss via recombination, requiring an extra filtering step at SIT facilities^{4,24}. Furthermore, males are semi-sterile due to associated aneuploidy, which limits the cost-effectiveness of the SIT programmes^{4,21,25}. To elevate GSS fitness, multiple transgenic approaches have been engineered for female exclusion either through a selectable phenotypic marker or female-specific lethality in the medfly and other fruit fly species^{26–36}. For example, a sex-specific intron from the *C. capitata transformer* (*tra*) gene has been implemented

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in the medfly for female-specific transgene expression from the autosomes, beginning with Fu et al.^{28,29,33}. Most recently, this was completed for female-specific fluorescence marker expression in a Sexing Element Produced by Alternative RNA-splicing of Transgenic Observable reporter (SEPARATOR) system, whereby both the *C. capitata* and, newly, the *A. ludens tra* introns were used²⁸. None of these systems, however, have been utilised at larger-scale facilities for release. This, in part, may be caused by the regulatory requirements on the release of transgenic insects, which vary regionally.

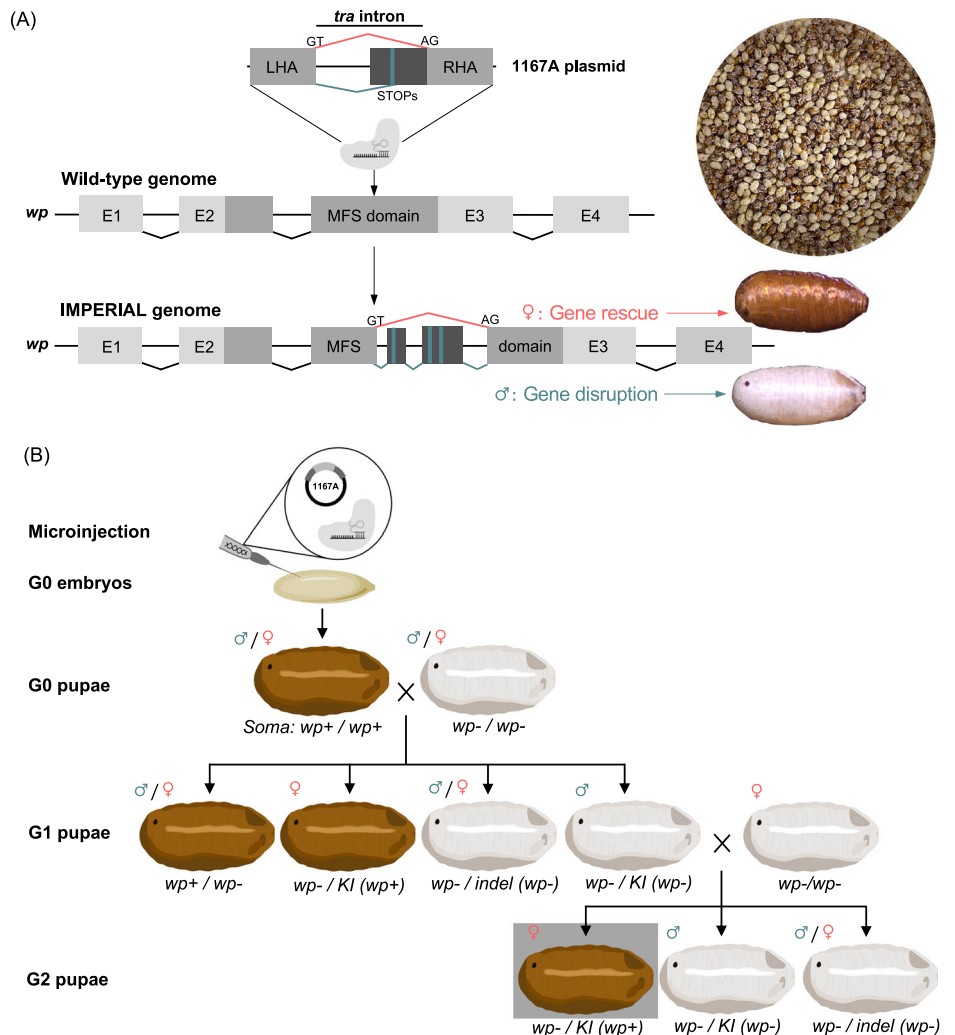
Herein, we wanted to develop a universal method in insects to engineer GSSs that use modern engineering techniques to generate minimal genetic modifications wherein both donor and recipient are derived from the same species, which we term Cisgenic GSS (CGSS). We anticipate that our cisgenic approach will provide a more palatable option for wider-scale implementation due to its vital characteristic of containing no foreign DNA. We used the medfly as our model system to engineer a non-transgenic CGSS without exogenous elements. Sex-specific expression of the endogenous *wp* selectable marker was achieved through sex-specific splicing of an inserted copy of its *tra* intron. This was attained through a homology-directed repair (HDR)-dependent knock-in of the *tra* intron seamlessly into the *wp* locus of wild-type Benakeion medfly embryos, feasible due to recent success of CRISPR/Cas9-mediated HDR in the species³⁷⁻³⁹. The key intention of the work presented herein, whereby *C. capitata* is used as a model, is to replicate CGSSs in other pests including tephritid pests with ongoing SIT programmes without pre-existing traditional GSSs of sufficient quality.

Results

Collectively, using the intron of *tra*, a master gene of tephritid female sex determination⁴⁰⁻⁴², results in extremely robust desired phenotypes, rendering it suitable for further use in CGSSs, yet to be developed. The knock-in construct (1167 A) was engineered using the endogenous *tra* intron, which was placed between the homology arms, each approximately 700 bp in length. The anticipated outcome was female-specific *wp* gene expression, whereby males harbouring two copies of the *tra*-containing *wp* gene would have a white pupae phenotype and females harbouring two copies of the *tra*-containing *wp* gene would have a brown pupae phenotype, which can be distinguished by eye (Fig. 1A). As the *wp* mutation is recessive in nature, the knock-in strain, hereupon named IMPERIAL, was isolated using backcrosses to the *white pupae* knock-out (*wp*^{-/-}) strain at G0 and G1 (Fig. 1B). We confirmed successful knock-in of the *tra* intron (*wp*^{KI/+}) in all obtained brown-pupaed G2 females using amplicon sequencing of the integration site. A homozygous *wp*^{KI/+} line was established at G7 (F0) after crossing sibling white-pupaed males with brown-pupaed females, genotyping all parents and screening the whole progeny at every intermediate generation. To verify the integration in the *wp*^{KI/+} IMPERIAL strain, genomic DNA from CGSS females was sequenced. The sequencing revealed reads with the expected 1345 bp CRISPR-HDR insertion, indicating a seamless intragenic insertion of the *tra* intron into the *wp* gene (Supplementary Fig. 1).

To verify the sex-sorting suitability of the strain, phenotypic pupae colour stability and sex ratios of the IMPERIAL strain were examined alongside the existing VIENNA 8 GSS, where males and females emerge from brown and white pupae, accordingly. For five consecutive generations

Fig. 1 | Design behind the cisgenic IMPERIAL strain. A A simplified diagram showcasing the IMPERIAL strain generation and its underlying mechanism. The knock-in, mediated via homology-directed repair was performed into the Benakeion wild-type strain. Due to the presence of premature stop codons in the male-specific exons, the males are phenotypically white-pupaed, whilst in females, gene rescue occurs resulting in a brown-pupaed phenotype (E1-E4, Exons 1-4; LHA, left homology arm; MFS, Major Facilitator Superfamily; RHA, right homology arm; *tra*, transformer; *wp*, white pupae). B Graphic summary of IMPERIAL strain establishment via outcrosses to the homozygous recessive *white pupae* mutant (*wp*^{-/-}) strain (KI, knock-in).



(F2-F6), pupae and adult phenotypes were recorded for IMPERIAL (pupae $n = 4147$; adult $n = 4074$) and VIENNA 8 (pupae $n = 1386$; adult $n = 1227$) strains in parallel, maintained under the same conditions. As anticipated, in the $wp^{KI/+}$ IMPERIAL strain, all females emerged from brown pupae and all males emerged from white pupae (Fig. 2A). The reverse phenotypes were universally observed amongst individuals from the VIENNA 8 strain. Whilst populations within every generation of the IMPERIAL strain adhered to the expected 1:1 male: female sex ratio, the VIENNA 8 did not at any of the five generations assessed (Fig. 2A). Binomial tests estimated overall male adult frequency of 0.506 (95% CI = 0.491, 0.522; $p = 0.443$) and 0.620 (95% CI = 0.592, 0.647; $p < 2.2e^{-16}$ *****) in IMPERIAL and VIENNA 8 strain populations, respectively. In sum, these results confirm the phenotypic stability and expected survival of both sexes in the IMPERIAL strain. In VIENNA 8, female lethality may be at play as the GSS did not consistently confine to the expected 1:1 male: female sex ratio.

To better understand differences in the *tra* intron-dependent *wp* splicing in males and females of the $wp^{KI/+}$ IMPERIAL strain, we performed reverse-transcription PCR (RT-PCR) on genomic and complementary DNA (cDNA) templates from adult flies (Supplementary Fig. 2). A single female band was amplified from the cDNA template, corresponding to a transcript with the fully spliced-out *tra* intron³⁸, confirmed by sequencing thereafter. Male cDNA banding entirely consisted of larger fragments from which two unique male isoforms with premature stop codons were isolated via clonal sequencing, both containing sequences of the two male-specific exons. These results are suggestive of functional White pupae protein production in females and ablation of its translation in males. Further investigation into *wp* splicing of the IMPERIAL strain was conducted using RNAseq. As expected, all three female libraries had multiple reads that spanned the junction splicing the *tra* intron. In contrast, the three male libraries had no such reads, indicating that the intron is spliced in females and not in males (Supplementary Fig. 3). As expected, the clustering and principal component analyses indicated a close relationship of samples by sex (Supplementary Fig. 4).

The IMPERIAL sexing strain was further compared to its parental wild-type Benakeion and VIENNA 8 strains in terms of general fitness, and thus suitability for larger-scale employment. First, a standard egg-adult survival assay utilising triplicate sibling crosses of 10 males with 20 females was conducted, whereby rates of egg laying, egg hatching, hatched larva-pupae recovery, pupae-adult recovery, and total egg-adult recovery were assessed (Fig. 2B). The data did not entirely conform to normal distribution; hence, Kruskal-Wallis and sequential Dunn's tests were performed for pairwise comparisons. Notably, egg production within the measured time period was significantly elevated in the IMPERIAL strain compared to both wild-type ($p = 0.0181^*$) and VIENNA 8 ($p = 0.0258^*$) strains. Hatching rate in VIENNA 8 (mean = 0.568, 95% CI = 0.413, 0.723) was significantly lower than in wild-type (mean = 0.821, 95% CI = 0.748, 0.894; $p = 0.0036^{**}$), although non-significant reductions in the IMPERIAL strain were also observed compared to wild-type (mean = 0.741, 95% CI = 0.660, 0.822; $p = 0.0899$). We recorded the highest larval-pupal survival in the IMPERIAL strain (mean = 0.950, 95% CI = 0.921, 0.978), which was significantly raised compared to VIENNA 8 (mean = 0.605, 95% CI = 0.297, 0.912; $p = 0.0036^{**}$). In line with the earlier phenotypic stability experiments, the pupal-adult recovery rates were significantly lower in the VIENNA 8 strain (mean = 0.882, 95% CI = 0.857, 0.907), compared to both wild-type (mean = 0.958, 95% CI = 0.915, 1.001; $p = 0.0368^*$) and IMPERIAL (mean = 0.958, 95% CI = 0.824, 1.091; $p = 0.0127^*$) strains. Overall egg-adult survival was significantly reduced in VIENNA 8 (mean = 0.301, 95% CI = 0.148, 0.454) when assessed against wild-type (mean = 0.691, 95% CI = 0.582, 0.800; $p = 0.0127^*$) and IMPERIAL (mean = 0.674, 95% CI = 0.573, 0.774; $p = 0.0368^*$) strains alike. Egg-adult survival difference between wild-type and IMPERIAL strains, however, was non-significant ($p = 0.3274$), consistent with the null hypothesis. Although given that the confidence intervals varied at different developmental stages, the sample sizes may limit the identification of any further potential strain-by-strain differences.

We also explored adult longevity with virgin males and females restricted to separate husbandry under regular lab conditions (Fig. 2C).

Triplicates of 10 males and 10 females were assessed, totalling 30 flies per sex per strain (total $n = 180$). Highest survival was observed in wild-type females, whilst the shortest longevity belonged to VIENNA 8 females. Altogether, strains were compared with and without consideration for sex using separate Cox PH models. Overall, strain was a significant predictor of survival outcomes (VIENNA 8-IMPERIAL: HR = 1.757, CI = 1.20, 2.57; $z = 2.919$, $p = 0.00351^{**}$; IMPERIAL-wild-type: HR = 0.833, CI = 0.579, 1.200; $z = -0.981$, $p = 0.327$; VIENNA 8-wild-type: HR = 0.512, CI = 0.347, 0.756; $z = -3.37$, $p = 0.00075^{***}$). Additionally, strain and sex were combined predictors of survival outcomes (Supplementary Table 1).

A delay in white-pupaed female development has been documented in the VIENNA 8 strain⁴³. To determine whether similar issues occur in the males of the IMPERIAL strain because of their white-pupaed phenotype, we compared pupal eclosion times from age-matched egg collections by sex. We used total eggs from 24-hr collections from triplicate cages for each strain (adult total $n = 1532$). The strain was a significant predictor of eclosion time (VIENNA 8-IMPERIAL: HR = 0.066, CI = 0.047, 0.093; $z = -15.48$, $p < 2e^{-16}$ ****); IMPERIAL-wild-type: HR = 0.794, CI = 0.714, 0.883; $z = -4.251$, $p = 2.12e^{-05}$ ****); VIENNA 8-wild-type: HR = 8.750, CI = 6.503, 11.770; $z = 14.32$, $p < 2e^{-16}$ ****) (Fig. 2D). Strain and sex were, similarly, combined predictors of eclosion outcomes (Supplementary Table 1). IMPERIAL males (mean = 18.51 days) were the fastest to eclose, followed by IMPERIAL females (mean = 18.59 days). The wild-type Benakeion strain was the second fastest with males and females eclosing at means of 18.60 and 18.75 days. Flies from the VIENNA 8 strain were the slowest to develop, with means of 20.11 and 24.00 days for males and females, accordingly. This, in part, can be attributed to the differences in acclimation of the both males and females from the VIENNA 8 strain to a newer laboratory environment compared to the other two strains. The female-specific delay in VIENNA 8, on the other hand, has been previously linked to another gene on chromosome 5, *slow development* (*sd*)⁴⁴.

The mating preferences of females towards the IMPERIAL, Benakeion and VIENNA 8 strains were assessed simultaneously. All males were placed collectively with females from the $wp^{-/-}$ strain. After mating, females were separated, and their progeny was independently screened at the pupal and adult stages of development. Due to the recessive nature of the *wp* mutation, the father(s) were easily 'revealed' through pupal colour and corresponding adult phenotype screening (Table 1). The experiment was repeated three times, each time using 90 females, for a total of 270 females placed for mating. Out of these, 176 females produced viable progeny. The most common parentage belonged to wild-type male(s) (36.93%), followed by equal parentage by IMPERIAL male(s) (28.41%) and VIENNA 8 male(s) (28.41%). We also observed offspring cohorts from single mothers with mixed-strain paternity at a low frequency (6.25%). This data indicates that the pupal colour of the IMPERIAL males does not disadvantage their mating success in respect to brown-pupaed VIENNA 8 males. As with the pre-existing GSS, however, the IMPERIAL males are still outcompeted by wild-type, which requires clarification on a wider scale.

Discussion

Here, we describe an entirely novel CRISPR/Cas9-generated CGSS method with proof of concept for the major agricultural pest *C. capitata* using the *wp* gene involved in pupal pigmentation¹⁸. Although multiple robust next-generation GSS approaches have been established in the medfly to date^{27–29,36}, the herein-established IMPERIAL strain is the first to exclusively encompass endogenous elements. Our approach uses the well-characterised sex-specific intron of *tra*, a gene responsible for female-specific fate induction in the *Tephritidae* fruit fly family and beyond, making it an appealing target for cross-species application^{45,46}. Importantly, the Y-chromosome-independent GSS approach highlighted herein is time-effective as it only requires comprehensible cross-completion for line establishment. Via CRISPR/Cas9-mediated HDR, we integrated the *tra* intron into the *wp* gene to achieve its expression in females exclusively, resulting in a brown-pupaed phenotype. Due to sex-specific splicing (Supplementary Fig. 2), males in the strain do not translate *wp* transcripts

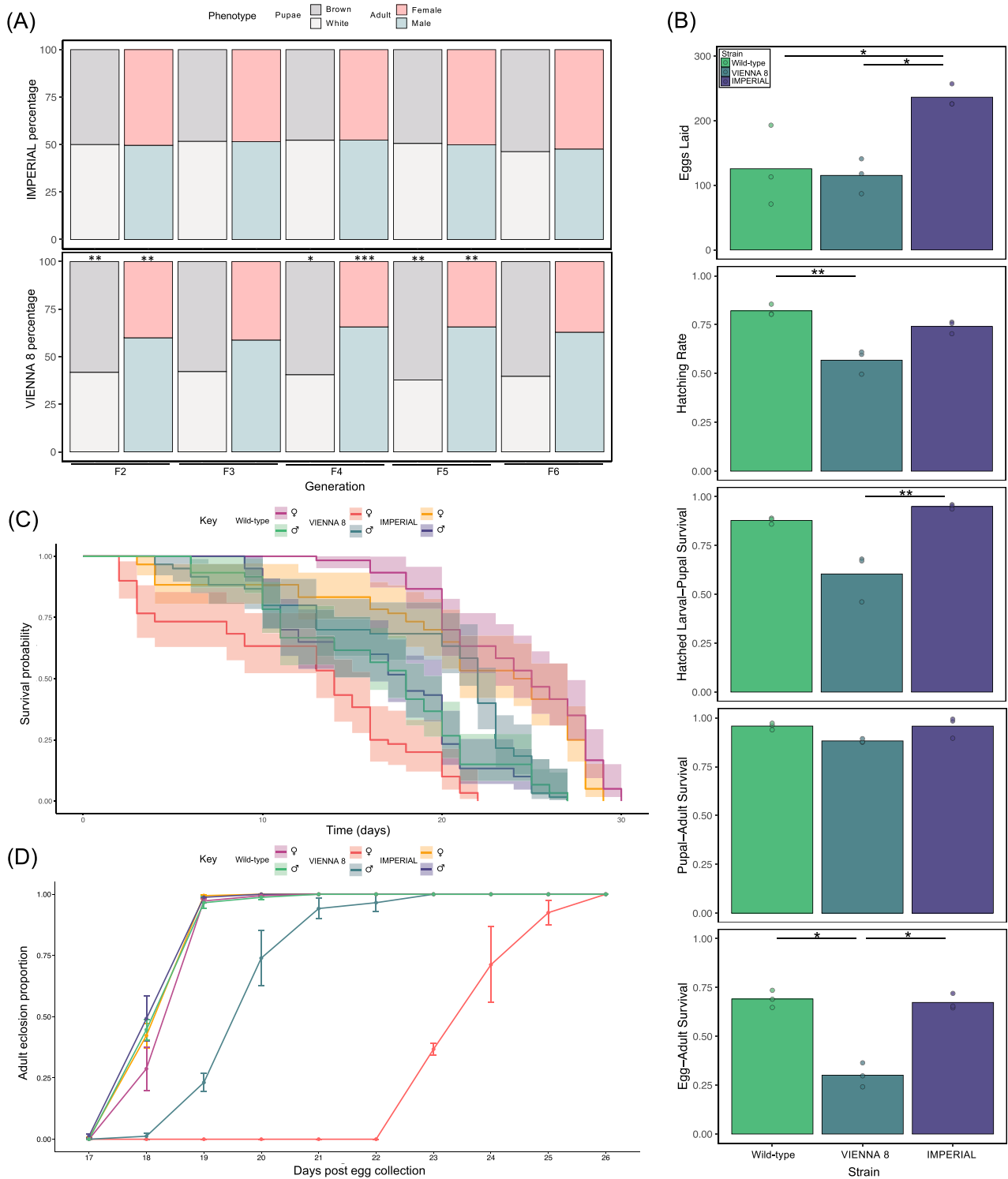


Fig. 2 | Characterisation of the cisgenic IMPERIAL sexing strain. **A** Stack graphs displaying pupal colour and adult phenotypes in IMPERIAL and VIENNA 8 strains for five consecutive generations (F2-F6). In the IMPERIAL strain, all males eclosed from white pupae and all females from brown. In the VIENNA 8 strain, the opposite phenotypes were universally observed. Binomial test significance levels are indicated as follows: $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$, $p < 0.0001 = ****$. **B** Bar charts showing egg-adult survival of the IMPERIAL strain compared to both its parental wild-type Benakeion, and VIENNA 8 strains, completed in biological triplicates. Egg-adult survival was measured using 5 h collections of eggs and their subsequent hatching rates, hatched larval-pupal recovery rates, and pupal-adult recovery rates.

Bar levels represent mean values, whilst individual replicate values are shown with dots. Dunn's test significance levels are indicated as follows: $p < 0.05 = *$, $p < 0.01 = **$. **C** Survival curves (Kaplan-Meier) of adult males and females from IMPERIAL, VIENNA 8 and wild-type Benakeion strains. The 95% confidence intervals are displayed using pale shading for each test group. **D** Proportion of eclosing males and females from IMPERIAL, VIENNA 8 and wild-type Benakeion strains measured daily from age-matched triplicate 24 h egg collections. Dots represent mean values of the replicates, and standard error is indicated using whiskers. **(A-D)** were constructed in RStudio. Source data is included in the Supplementary Data file.

Table 1 | Mating preference of females towards wild-type, IMPERIAL and VIENNA 8 strains

Paternity	Replicates			Total count	Percentage (%)
	1	2	3		
Wild-type male(s)	25	16	24	65	36.93
IMPERIAL male(s)	17	15	18	50	28.41
VIENNA 8 male(s)	13	18	19	50	28.41
Wild-type + IMPERIAL male(s)	0	0	0	0	0
Wild-type + VIENNA 8 male(s)	1	0	2	3	1.70
IMPERIAL + VIENNA 8 male(s)	2	3	3	8	4.55
Total	58	52	66	176	100

Males from the three tested strains were collectively released to mate with females from the recessive homozygous *white pupae* mutant (*wp*^{-/-}) strain in three repeats. Adult progeny from individual females was scored by pupal colour and corresponding adult phenotypes.

successfully. Specifically, this is caused by the inclusion of early termination codons upon transcription, leading to a white-pupae phenotype. Given that no foreign DNA is inserted into the genome, our approach may be easier to gain regulatory approvals for release, but this remains to be determined.

Characterisation of the IMPERIAL strain revealed that its fitness is comparable to its ancestral wild-type strain from which it was generated. This included survival during development and upon adulthood, as well as eclosion time comparison between males and females (Fig. 2). Despite statistically similar egg-adult survival, however, the IMPERIAL strain egg hatching rates were reduced non-significantly. In all the above-mentioned assessments, the VIENNA 8 strain performance indicated greater fitness costs than in the herein-generated strain, in part due to the male aneuploidy-driven semi-sterility and the anticipated female-specific developmental delay^{4,44}, although mating competitiveness was similar between the two GSSs (Table 1). This cannot be overlooked, as male competitiveness directly reflects the potential success of sterilised males upon release, corresponding to the efficiency of SIT. The IMPERIAL males, which were outcompeted by wild-type, need to be further tested at facilities against wild populations, instead of relatively inbred laboratory-housed strains. Additionally, IMPERIAL and VIENNA 8 strains have differential ancestral strains and hence genetic backgrounds, which ought to be taken into consideration regarding fitness parameters. Furthermore, great sample sizes, ideally on a broader scale, may be necessary to explore any further potential differences between strains, as the obtained data were variable in select experiments. To explore the fitness of the IMPERIAL strain in further detail, and hence its suitability for SIT implementation, larger-scale trials still need to be performed, which will also aid in understanding potential variations within strain performance. This is particularly important when comparing any strain to VIENNA 8, as it is a GSS fine-tuned for mass production. Among all males and females screened in the process of line characterisation in this study, no reverse phenotypes were observed, suggestive of line stability in the laboratory setting. Once more, larger-scale assessments are necessary to investigate whether a minimal genomic modification used herein would be more stable than the translocation-dependent VIENNA 8. Furthermore, similarly to wild-type flies, the males and females are consistently distributed in equal proportions in the IMPERIAL strain (Fig. 2A). This highlights the limited effect of the white-pupae phenotype on the male survival to adulthood in our GSS. However, it cannot be overlooked that the white-pupae IMPERIAL males may be disadvantaged from WT males in terms of mating, as observed in the *wp* mutants in *B. dorsalis*⁴⁷. As the expression patterns of the *wp* orthologue in *Drosophila melanogaster*, CG14439, are not limited to pre-pupation stages of larval development, the function of the *wp* gene may not be exclusively limited to pupal pigmentation⁴⁸. Hence, the *wp* knock-out may restrict its other potential functions, limiting their mating success of the IMPERIAL males. Male-specific rescue of the *wp* gene for its WT expression in the released males may prevent this from happening.

To transfer the CGSS approach to other tephritids, it is important to emphasise that the desired outcome for any GSS is traditionally a wild-type male phenotype. The CGSS, generated here, opposes this outcome. On the other hand, if a similar pupal colour-only GSS is established in related tephritids, the mutant phenotype-exhibiting males can be easily salvaged in case contamination with wild-type or alternative strains is detected at facilities during pupal screening. Prior to establishment, however, *wp* mutant strains ought to be isolated or generated through CRISPR in the species of interest. In theory, the sex-sorting of such CGSS strains can be performed at the pupal developmental stage using automated machinery, already employed and optimised at SIT *C. capitata* facilities. Thus, with its stability and cisgenic nature, our system could be an advantageous and cost-effective alternative for currently used strategies in SIT-mediated population control once transferred to other related tephritids currently without GSSs. The *C. capitata* proof-of-principle work presented in the current study is thus directly intended for this outcome.

The latest reiteration of the traditional GSSs in *C. capitata*, VIENNA 8, possesses a heat sensitivity component via the *tsl* gene, which aids in cost-effectiveness and effectiveness of the *C. capitata* SIT programmes as females do not need to be reared until pupation¹⁷. As its wild-type copy is exclusively expressed from the Y-chromosome-linked chromosome 5 translocated region, only males are tolerant to heat⁴. To improve our system further, a heat-inducible component can be added to the IMPERIAL strain for its male-specific expression. This will additionally allow for a thorough investigation of the fitness costs in VIENNA 8 observed in this work. Since the *tsl* locus has just been characterised in full¹⁹, alternative iterations of the *tra* intron for functional male-specific splicing, or entirely different sex-specific introns^{40,49}, can be used for its rescue. As a specific substitution mutation is associated with temperature sensitivity, the male-specific rescue could be placed into a different genomic location, ideally associated with null fitness costs. If a site-specific knock-in approach is used, the system would still be considered cisgenic. Female-specific heat sensitivity during embryogenesis would thus ensure that only males are reared till adulthood for release, a feature which the IMPERIAL strain presently lacks.

Materials & methods

Plasmid design and construction

We used Gibson enzymatic assembly to build the 1167 A plasmid, which contains *C. capitata* *MFS transporter* aka *wp* Exon 3 (LOC101451947), the *tra* intron and Opie2-DsRed outside the homology arms. A pre-existing plasmid containing *piggyBac* flanks, with an Opie2 promoter regulating DsRed, was linearised by NdeI and KpnI to clone 1167 A. The *MFS transporter* Exon3 was amplified into two fragments from *C. capitata* genomic DNA using primer pairs 1167 A.c1F and c2R, as well as 1167 A.c5F and c6R (Supplementary Table 2). The *tra* intron was amplified from 795H1 (Addgene #205482) using primer pair 1167 A.c3F and c4R, then inserted inside of the *MFS* Exon 3 coding sequence (Supplementary Table 2).

C. capitata maintenance

All fly strains were reared under standard lab conditions described previously⁵⁰. A carrot-based diet was provided for larval development²⁰ and a 1:1 yeast: glucose mix was given to adult flies. The Benakeion wild-type strain was supplied by the Saccone Lab (University of Naples "Federico II"), whilst *white pupae* *-/-* (*wp*^{-/-})⁵¹ and D53 inversion-less VIENNA 8 strains were obtained from the FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture (Seibersdorf, Austria).

C. capitata germline transformation

Microinjections of the 1167 A plasmid were performed into embryos of the wild-type Benakeion strain using the FemtoJet injection system (Eppendorf) with pulled quartz needles (World Precision Instruments). The plasmid (300 ng/μl) was injected alongside a pre-assembled ribonucleoprotein (RNP) complex of Cas9 protein (360 ng/μl) (PNA Bio) and pre-synthesised gRNA-*wp* (340 ng/μl) (Synthego)³⁹.

***wp*^{KI/+} line establishment**

The cross scheme for G0-G3 line generation is summarised in Fig. 1. The injected G0s were reciprocally crossed to the characterised *wp*^{-/-} strain. The resulting G1 progeny was separated by pupal colour, and all males emerging from white pupae were backcrossed in pools to the females from the *wp*^{-/-} strain. At G2, the brown pupae were isolated, and all 10 female adults from the same parental G1 cross were collectively crossed to five sibling white-pupaed males. After mating, genomic DNA (gDNA) was individually extracted from G2 brown-pupaed females using an altered phenol-chloroform protocol⁵². The knock-in site was amplified using Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs®) with genome-specific 1167A_F and 1167A_R primers (Supplementary Table 2) designed in Geneious Prime 2023.1.2. To confirm the initial integration, the PCR products were purified via Monarch® PCR & DNA Cleanup Kit (New England Biolabs®) and analysed via Oxford Nanopore sequencing (Full Circle Labs).

Over the course of the following 4 generations (G3-G6), multiple sibling crosses were performed in parallel, and the parental genotypes were verified. Hereby, three possible alleles were differentiated: 1) the backcross *wp*^{-/-}; 2) the intron-less *wp*^{-/-} with indels and 3) the *tra* intron knock-in (Supplementary Fig. 5). For this, a multiplex 3-primer PCR was designed using a forward (1167A_F) binding upstream of the integration site, a reverse (1167A_R) downstream of the integration site and a second reverse (8kb_B_R) binding to the *wp*^{-/-} inserted sequence (Supplementary Fig. 5).

Verification of genome integration site in the homozygous strain

To verify the insertion site in the *wp*^{KI/+} IMPERIAL strain, we conducted Oxford Nanopore genomic DNA sequencing. Genomic DNA was extracted from four knock-in adult males and four knock-in adult females using the Blood & Cell Culture DNA Midi Kit (Qiagen).

PCR *tra* intron splicing confirmation

In parallel, single males and females were separately collected for gDNA and RNA extractions. gDNA was extracted as detailed above. RNA was extracted via an adapted TRIzol® (Ambion)-chloroform-based protocol⁵³. cDNA was synthesised from total RNA using Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (ThermoFisher) according to the instructions provided by the manufacturer. Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs®) was used for PCR amplification from gDNA and cDNA templates using the 1167_F_V1 splicing and 1167A_R primer pair (Supplementary Table 2). Bands amplified from cDNA of the IMPERIAL strain were purified using a Monarch® DNA Gel Extraction Kit (New England Biolabs®) and Sanger sequenced (Genewiz Inc.). Male cDNA PCR reaction was additionally subjected to Sanger sequencing post-PCR product cloning using the StrataClone PCR Cloning Kit (Agilent) whereby different isoforms were isolated.

RNA sequencing

To confirm the sex-specific expression of the *wp* gene, we performed Illumina RNA sequencing. Total RNA was extracted from mature *wp*^{KI/+} adult males and females from the IMPERIAL strain in three biological replicates (six samples) using the miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen), following the manufacturer's protocol. Genomic DNA was removed using the gDNA eliminator column included with the kit. RNA integrity was tested with the RNA 6000 Pico Kit for Bioanalyzer (Agilent Technologies).

Male (ID# 27026-27028) and female (ID# 27023-27025) libraries with three replicates each were sequenced to an approximate depth of 20 M paired-end reads (Supplementary Data 2). The reads were aligned with STAR to the EGII-3.2.1 genome assembly (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_905071925.1/), into which *traF* intron was inserted at the white pupae locus (GCA_905071925.1_EGII-3.2.1_genomic.traF-intron.fna). To generate a more complete annotation file, we transferred the Ccap_2.1 annotations (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_000347755.4/) to the EGII-3.2.1 genome by aligning Ccap_2.1

transcript sequences with BLAT and parsing the alignments to generate a GTF file (Supplementary Data 1), which was used for all subsequent analysis steps.

Gene abundances were quantified with featureCounts (<https://subread.sourceforge.net/featureCounts.html>), count data (Supplementary Data 3) were converted to TPM (Supplementary Data 4) and FPKM (Supplementary Data 5) values and combined using Perl scripts. Gene annotations were downloaded from EnsemblMetazoa using the BioMart tool (https://metazoa.ensembl.org/Ceratitis_capitata_gca000347755v4/Info/Index) and added to the quantification data (Supplementary Data 3-5). TPM values were used to perform PCA and clustering analyses in R to identify possible sample outliers. Replicates for each sex clustered together as expected and displayed high correlations between each other without obvious outliers (Supplementary Fig. 4, Supplementary Data 6). To visualize splicing of the *traF* intron within the white pupae locus (LOC101451947), BAM files produced by STAR were imported into IGV (Supplementary Fig. 3).

Stability assay

From G3 until G7 all pupae were separated by colour and corresponding adults were scored by sex. When the homozygosity of the IMPERIAL strain was verified at G7 (F0), alongside the VIENNA 8 strain, for 5 consecutive generations (F2-F6) eggs were collected five days after eclosion and raised under regular conditions until pupal stage of development. Brown and white pupae were then separated, and the sex of all adults from each pool was recorded.

Egg-adult survival assay

Crosses of 10 males and 20 females originating from the same crosses of pooled siblings were set up in triplicates simultaneously for the IMPERIAL (F4 generation), VIENNA 8, and wild-type Benakeion strains. Egg numbers and their hatching rates were determined as described previously²⁸. Specifically, five days after eclosion, all eggs laid within a 5-hour period were collected and unhatched eggs were counted twice four days apart using Fiji⁵⁴. Pupal and adult recovery rates were determined thereafter.

Adult longevity assay

Age-matched adults from the wild-type Benakeion, IMPERIAL (F5 generation) and VIENNA 8 strains were separated by sex upon eclosion and placed into cages of 10 individuals. Three male and three female replicates were set up simultaneously for each strain; and maintained under standard conditions thereafter. They were provided with sources of water and food consisting of yeast and glucose, both of which were replenished regularly. Daily, dead flies were counted and removed from the cages for 30 consecutive days.

Mating-preference assay

90 females from the *wp*^{-/-} strain were simultaneously placed together with 15 males from each of the Benakeion, IMPERIAL and VIENNA 8 strains for a total of a 1:2 male:female ratio. The experiment was repeated 3 times. The flies were left to mate for 4 full days, after which females were separated into individual small cages. Upon oviposition, eggs from all females which oviposited were separately collected and reared normally until pupation. In the cases where white and brown pupae were present, they were separated by colour. Adults eclosing from both mixed and brown pupae-only collections were screened by sex. IMPERIAL-only parentage was established when all males and females eclosed from white and brown pupae, respectively, whilst VIENNA 8 parentage was determined if the opposite was true.

Eclosion assay

24 h egg collections were made from sibling crosses of 10 males and 20 females of the IMPERIAL (F4 generation), VIENNA 8, and wild-type Benakeion strains, set up in parallel triplicates. The offspring were reared under normal conditions until pupation, whereby IMPERIAL and VIENNA 8 pupae were sorted by colour. The eclosing adults were therein scored by sex every day.

Figure generation and statistical analyses

All statistical analysis and plot generation were performed in R (versions 4.4.1 and 4.4.2) using the RStudio extension. Binomial tests were used for sex ratio and pupal colour analysis. Egg-adult survival was assessed using Kruskal-Wallis and Dunn's tests after an initial verification of normality using the Shapiro-Wilk test. Longevity data was plotted and analysed using survival and survminer packages. Ecllosion rates and longevity data sets were analysed with Cox PH models, separately established for each comparison. Microsoft PowerPoint and Inkscape 1.3.2⁵⁵ were used to create construct and fly-centred diagrams.

Data availability

Complete plasmid sequence is available at Addgene.org (#218233). The raw data used for figure generation are provided in the Supplementary Data 1–6. The sequencing data are available at NCBI under BioProject PRJNA1189200 (Reviewer link <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1189200?reviewer=8am21kg9tm2eskhv6ls27knsd>). The herein-generated knock-in strain is available upon request from A.M.

Code availability

The code is available at: <https://zenodo.org/records/15830371>.

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Author contributions

A.M. and O.S.A. conceived the project and directed the research with contributions from E.B. J.L., and N.P.K. designed the construct and J.L. performed the cloning. A.M. and O.S.A. designed the experiments. A.M. performed the germline transformation. S.D. performed medfly and molecular validation experiments. S.D. maintained the strains with the help of J.M. A.M., S.D., and O.S.A. analysed the data. J.L. prepared samples for sequencing; I.A. performed the sequencing and analysis. S.D. has written the first draft of the paper with additional contributions from A.M, J.L., I.A., and O.S.A. All authors contributed to the subsequent editing of the manuscript and approved the final article.

Competing interests

The authors declare the following competing interests: A patent has been filed on this technology PCT/US2025/061221. O.S.A is a founder of Agragene, Inc. and Synvect, Inc. with equity interest. N.P.K is a founder of Synvect, Inc. with equity interest. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict-of-interest policies. All other authors declare no competing interests.

Additional information

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